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Effects of Chlorinated Seawater on Decapod Crustaceans and 'Mulinia' Larvae

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Effects of Chlorinated Seawater on Decapod Crustaceans and Mulinia Larvae

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Eggs and larvae of decapod crustaceans and embryos of *Mulinia lateralis* were exposed to chlorinated seawater for varying periods in continuous flow systems. Mortality, developmental rate, and general behavior were recorded. *Panopeus herbstii* zoeae were more sensitive to chlorine-induced oxidants (CIO) than eggs or adults (96-hr LC50 ca. 2.8 μeq/l ≡ 0.1 mg/l). The 96-hr LC50 for *Pagurus longicarpus* zoeae was approximately the same as for *Panopeus* zoeae. The 120-hr LC50 for *Pagurus* zoeae was 1.4 μeq/l (0.05 mg/l). Development was slightly delayed for *Pagurus* zoeae at CIO levels as low as 0.6 μeq/l (0.02 mg/l). *Mulinia* embryos exposed for 48-hr had an LC50 between 0.3 and 2.8 μeq/l (0.01 and 0.1 mg/l). *Mulinia* embryos exposed to chlorinated seawater for 2-hr had an LC50 of about 2.0 μeq/l(0.072 mg/l); subsequent survival rates for larvae in unchlorinated seawater were unaffected by prior exposure to CIO.

The effects of CIO on serum constituents in *Callinectes sapidus* occurred sporadically and appeared unrelated to dose or mortality. Similar effects were noted for oxygen consumption in whole crabs and excised gills. It was concluded that there are no physiologically significant sublethal effects of CIO on serum constituents (osmoregulation) or oxygen consumption of whole blue crabs or excised gills. Blue crab antennules are sensitive to sublethal doses of CIO. Spawning and feeding are inhibited by sublethal doses of CIO. This effect disappears when CIO is removed.

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EFFECTS OF CHLORINATED SEAWATER ON DECAPOD CRUSTACEANS AND MULINIA LARVAE

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FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying conditions is required. The EPA Environmental Research Laboratory, Gulf Breeze, and its field station, Bears Bluff, contribute to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystem processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments.

This report presents results of bioassay tests of chlorination-induced oxidants upon larval crabs, blood physiology of the commercial blue crab, and toxicity to a small estuarine clam that enters the crab's food web. In addition to the effects measured with these organisms, the experimental apparatus described is relevant to future tests on low levels of stress upon the sensitive life stages of marine estuarine organisms. These data have appeared in part in refereed scientific journals and symposia, and should contribute substantially to our collective analysis of biological risks of oxidative products from chlorination discharged into marine waters.

Thomas W. Duke

Thomas W. Duke
Director
Environmental Research Laboratory
Gulf Breeze, Florida

ABSTRACT

Eggs and larvae of decapod crustaceans and embryos of *Mulinia lateralis* were exposed to chlorinated seawater for varying periods in continuous flow systems. Mortality, developmental rate, and general behavior were recorded. *Panopeus herbstii* zoeae were more sensitive to chlorine-induced oxidants (CIO) than eggs or adults (96-hr LC50 ca. 2.8 µeq/l ≅ 0.1 mg/l). The 96-hr LC50 for *Pagurus longicarpus* zoeae was approximately the same as for *Panopeus* zoeae. The 120-hr LC50 for *Pagurus* zoeae was 1.4 µeq/l (0.05 mg/l). Development was slightly delayed for *Pagurus* zoeae at CIO levels as low as 0.6 µeq/l (0.02 mg/l). *Mulinia* embryos exposed for 48 hr had an LC50 between 0.3 and 3.0 µeq/l (0.01 and 0.1 mg/l). *Mulinia* embryos exposed to chlorinated seawater for 2 hr had an LC50 of about 2.0 µeq/l (0.072 mg/l); subsequent survival rates for larvae in unchlorinated seawater were unaffected by prior exposure to CIO.

The effects of CIO on serum constituents in *Callinectes sapidus* occurred sporadically and appeared unrelated to dose or mortality. Similar effects were noted for oxygen consumption in whole crabs and excised gills. It was concluded that there are no physiologically significant sublethal effects of CIO on serum constituents (osmoregulation) or oxygen consumption of whole blue crabs or excised gills. Blue crab antennules are sensitive to sublethal doses of CIO. Spawning and feeding seem to be inhibited by sublethal doses of CIO. This effect disappears when CIO is removed.

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SECTION I

INTRODUCTION

Chlorine has been used extensively in the United States and elsewhere as a disinfectant for treated sewage. More recently it has come into vogue as an antifouling agent for once-through power plant cooling systems. The methods of application, disinfectant properties, and chemistry are extensively reviewed (White, 1972). It has been considered effective in both applications and was believed to be environmentally safe because the products formed upon chlorination of water are highly unstable; hence, according to traditional reasoning, troublesome concentrations would not be found in receiving waters under normal operating conditions.

The chemistry of chlorine in fresh water has recently been reviewed (White, 1972; Brungs, 1974; and Jolley, 1973). The descriptions provided therein do not, however, apply directly to salt water. Salt water contains sufficient bromide to allow conversion of chlorine compounds to analogous bromine compounds (Dove, 1970; Sugam and Helz, 1977; Johnson, 1977). Reactions with ammonia and organics can still occur. Macalady et al. (1977) have recently demonstrated bromate formation in chlorinated seawater exposed to sunlight. There remains considerable uncertainty concerning the halogen species present in chlorinated seawater since the analytical methods do not discriminate between the possible compounds present. Hence one cannot state positively what chemical species are causing observed toxic effects. Throughout this report the concentration of measured toxicant is referred to as chlorine-induced oxidant (CIO), expressed as μeq/l (mg Cl_2/l), in an attempt to avoid confusion regarding the active material.

The environmental safety of chlorine came into serious question during the 1960's. Zillich (1970) reported adverse effects of chlorinated sewage on aquatic life in fresh water systems. Subsequently, Bellanca and Bailey (1977) (previously reported in Va. State Water Control Board, 1974) described data which strongly implicated chlorine residuals emanating from sewage treatment plants as the causative agent of massive fish kills in the estuarine portion of the James River in Virginia. Hamilton et al. (1970), Morgan and Stross (1969), and Carpenter et al. (1972) found effects on natural phytoplankton exposed to chlorinated power plant effluents attributable to chlorine.

An extensive literature, describing the toxicity of chlorine in both fresh and estuarine waters to phytoplankton, various invertebrates, and fishes, has been amply reviewed and discussed by Brungs (1973, 1976). Toxicity studies relating to estuarine species have been very limited compared to those for freshwater species. Published studies deal primarily with acute lethal effects.

From our studies in Virginia, oyster larvae (*Crassostrea virginica*) and the copepod (*Acartia tonsa*) appeared to be the most sensitive estuarine species (48-hr LC50's of 0.73 and 0.82 μeq/l = 0.026 and 0.029 mg/l), while *Palaemonetes pugio* was most tolerant (96-hr LC50 = 6.20 μeq/l = 0.22 mg/l). Fishes tested were intermediate in sensitivity (Roberts et al., 1975; Roberts and Gleeson, 1978). Phytoplankton exposed to chlorine exhibited marked reductions in primary production in tests with cultured single-species populations (Roberts and Diaz, 1976, unpublished manuscript; Bender et al., 1977) and natural mixed populations (Roberts and Illowsky, unpublished data).

There has been little or no research on chronic effects of chlorine on estuarine organisms. Long-term exposure chronic studies have been carried out in freshwater with *Daphia magna*, *Gammarus pseudolimnaeus* and *Pimepheles promelas* (Arthur et al., 1975). Such studies have yet to be performed with estuarine species.

For the acute and sublethal tests in our study, we selected species representative of the decapod crustaceans and bivalve molluscs in Atlantic coastal estuaries. From the decapods, we selected the hermit crab, *Pagurus longicarpus*, and the mud crab, *Panopeus herbstii*, because they are abundant and widely distributed in estuaries, and the larvae can be readily cultured in the laboratory at the ambient salinity available to us. Further, *P. longicarpus* had previously been induced to spawn in the laboratory during the winter (Roberts, 1969). Since these species are characteristic of the estuarine fauna, they are presumed to be of ecological significance. The shrimp *Palaemonetes pugio* was not selected because of the high adult tolerance (Roberts et al., 1975). The blue crab *Callinectes sapidus* was not selected because larval salinity requirements (25 °/oo or above) could not be readily provided in our laboratory. As a representative bivalve, the coot clam, *Mulinia lateralis*, was selected. *M. lateralis* is an abundant representative of the estuarine infauna, capable of completing its life cycle in 6 to 9 months under appropriate conditions. Its value for toxicological studies has previously been pointed out by Calabrese (1969b, 1970).

Historically, studies of the physiological effects of exposure of aquatic animals to chlorine residuals have been limited to fish.

Typically, the effects have been manifested as changes in properties of the blood such as methemoglobin and lactate concentrations, pO_2, pH, hematocrit, and plasma water as well as erythrocyte condition and maturity. Because of basic physiological differences, the relationship between the effects in fishes and those in invertebrates is uncertain. Accordingly, we elected to investigate the effects of chlorine residuals on certain blood and respiratory parameters in the blue crab, Callinectes sapidus. The blue crab was chosen because it is a commercially important, common, active inhabitant of the Chesapeake Bay and its tributaries and is likely to encounter chlorinated effluents from sewage and power plants. In addition, the crab offers the availability of a large physiological data base.

Voluminous literature offers useful physiological information derived from measurements of crab blood parameters. Blue crab blood, already used to assess crab responses to temperature, salinity and season, pesticides, disease, and general stress, has provided information on mechanisms of ionic and osmotic accommodation and respiration. Changes in blue crab physiology caused by chlorine-induced oxidants should also be reflected in the blood and could probably be observed in the serum constituents.

A portion of this study was conducted to establish the effects of exposure to chlorine residuals on serum constituents in the blue crab. Serum constituents were used in order to develop a physiological index for assessing and estimating the degree of stress imposed on crabs by different levels of residuals - a possible alternative to long-term bioassays.

Since the gill is the primary interface across which blue crabs interact with their environment, it is reasonable to suspect that physiological effects of chlorine induced oxidants on blue crabs originate in the gill. Effects on osmoregulatory sites in the gill should be manifested in the serum constituent studies. In fishes it has been concluded that the primary mode of action of chlorine is gill tissue damage resulting in death by asphyxiation. If gill damage occurs in the blue crab on exposure to chlorine-induced oxidants, death by asphyxiation is likely. Part of the study was devoted to this problem. Specific attention was afforded to the effects of chlorine-induced oxidants on whole crab oxygen consumption, excised gill oxygen consumption and gill histopathology.

The objectives of the present study were;

1. to assess the subacute effects of chlorinated seawater on Panopeus herbstii and Pagurus longicarpus eggs and larvae, and larvae of the coot clam, Mulinia lateralis.

2. to determine whether Pagurus longicarpus larvae can avoid chlorinated seawater,

3. to examine the effects of exposure to chlorinated seawater on the blood chemistry of blue crabs at sublethal doses and, concurrently, to determine the time course of blood serum changes,

4. to examine the effects of exposure to chlorinated seawater on the oxygen consumption rate of whole blue crabs and blue crab gills.

SECTION 2

CONCLUSIONS

1. The sensitivity to chlorinated seawater for Panopeus herbstii varies as a function of life history stage. Panopeus herbstii eggs can develop to hatching at CIO levels up to 5.9 μeq/l (0.21 mg/l), whereas the resultant larvae at this dose exhibit less than 10% survival. The 96-hr LC50 for Panopeus herbstii larvae varied from 1.13 to 3.38 μeq/l (0.04 to 0.12 mg/l) in most tests. Larvae tested early and late in the breeding season seemed to be somewhat more sensitive with a LC50 of ca. 0.68 μeq/l (0.024 mg/l). In a preliminary test with field-collected juveniles, the 96-hr LC50 was 14.1 μeq/l (0.50 mg/l).

2. The larvae of Pagurus longicarpus exposed to chlorinated seawater exhibited a 96 hr LC50 of 1.75 to 2.88 μeq/l (0.062 to 0.102 mg/l), which is similar to the LC50 for Panopeus herbstii larvae.

3. Sublethal doses of chlorinated seawater caused a delay in development of Pagurus longicarpus larvae.

4. Mulinia lateralis embryos exposed to chlorinated seawater for the first 48 hr after fertilization exhibited an LC50 between 0.3 and 2.8 μeq/l (0.01 and 0.10 mg/l).

5. Mulinia embryos exposed to chlorinated seawater for 2 hr after fertilization exhibited an LC50 of about 2.0 μeq/l (0.072 mg/l). During post-exposure culture in static unchlorinated water, larvae from all treatments exhibited approximately equivalent survival rates.

6. There are no physiologically significant sublethal effects of CIO on serum constituents (osmoregulation) or oxygen consumption of whole Callinectes sapidus or excised gills.

7. CIO in water irritates Callinectes sapidus, causing retraction of the first antennae bearing sensory sites. Further, we observed reduced feeding and no spawning among crabs exposed to CIO. Feeding was restored when CIO was removed.

SECTION 3

RECOMMENDATIONS

1. Studies of both inorganic and organic chemical speciation of chlorinated saline water are needed in order to identify more specifically those chemicals responsible for observed biotic effects. Variations in observed LC50 values expressed in terms of total CIO may derive from differences in the specific chemicals present at various pH and ammonia levels.

2. Research should be designed specifically to examine seasonal changes in CIO tolerance and differences in tolerance that might be anticipated at estuarine locations of differing salinity.

3. The potential for crabs to recover from short-term exposure to lethal doses of CIO (i.e. 96-hr LC50 and above) needs to be evaluated from a physiological and histological perspective.

4. The physiological mechanism of CIO-induced mortality in blue crabs remains to be ascertained. Knowledge of the mechanism is important to the evaluation of possible sublethal physiological effects.

5. Improved exposure methods and test protocols need to be developed for Mulinia (and other bivalve) larvae challenged by chemically unstable pollutants. Attention should be given especially to culture requirements of volume, larval density, and enumeration techniques.

6. Quantitative studies of CIO effects on avoidance behavior, fecundity, spawning, feeding and sensory activities of the blue crab would be useful to better evaluate the overall ecological impact of CIO on this species.

7. Histological examinations should be made on selected tissues of adult crabs in an attempt to identify the organ systems affected by CIO. Tissues of greatest interest are gills, mid-gut, aesthetascs, hepatopancreas, and gonads.

8. **The data on biotic effects of ClO at very low concentrations strongly suggest the need to direct more future research to alternatives to chlorination, including chlorination/dechlorination, bromochlorination, and ozonation.**

SECTION 4

DEVELOPMENTAL STUDIES

GENERAL METHODS

Dosing Apparatus

In order to expose all life stages of the crab species and molluscan larvae to reasonably uniform concentrations of the chemically unstable toxicants, chlorine induced-oxidants, it was necessary to utilize a continuous flow system. The dosing system built for the experiments described herein is diagrammatically represented in Figure 1.

Diluent water was pumped from the York River estuary at Gloucester Point, Virginia, through PVC pipe to the bioassay laboratory. The water was passed through 10 µm or 5 µm GAF filter bags (A) and collected in a reservoir (B). This water was then pumped (C) through 1 µm Honeycomb filters (PVC core) in parallel or 10 µm and 1 µm filters (E) in series into a diluent header tank (F). Unused filtered water was returned to the reservoir.

The diluent was delivered to a series of mixing chambers (G) by means of siphons calibrated for short intervals (hrs) to deliver a specified flow rate ± 1% (approx.) at a given head. Chlorine was introduced into the mixing chambers by injecting stock solutions of $Ca(OCl)_2$ (I_1 to I_5) with a Harvard peristaltic pump (H) at a rate of 1 ml/min. The mixed solution was then delivered to the test chambers through glass delivery tubes.

A float was installed in each header tank so that if the diluent water level fell beyond 5-10 mm, a microswitch was opened, interrupting all power to the toxicant (H) and diluent water (C) pumps. Heaters located in the header tanks during the winter to maintain the desired temperature were protected by the same system to prevent damage to equipment.

Flow rates of toxicant stock and diluent were measured three times daily during all experiments. Table 1 presents the data from one experiment which extended over an 18-day period. The mean

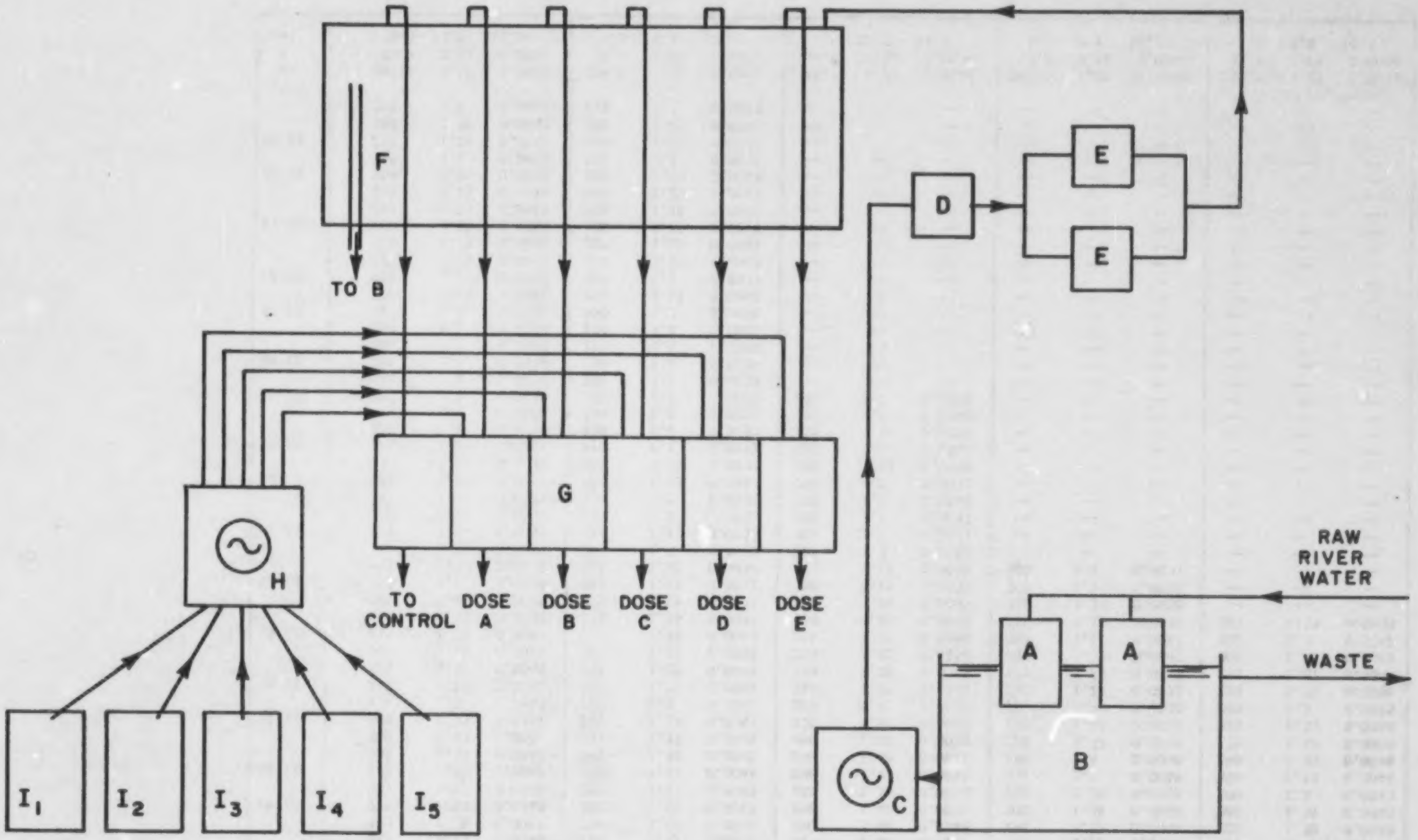
F
TO B
G
H
TO CONTROL
DOSE A
DOSE B
DOSE C
DOSE D
DOSE E
I1
I2
I3
I4
I5
D
E
E
C
A
A
B
RAW RIVER WATER
WASTE

Figure 1. Schematic representation of dilutor system.

TABLE 1. SUMMARY OF FLOW RATE DATA (DILUENT AND TOXICANT STOCK) AND CALCULATED DILUTION FACTORS FOR LARVAL EXPERIMENT 9

Dose Level/	A			B			C			D			E		
Date	Dil.	Stock	DF	Dil.	Stock	DF	Dil.	Stock	DF	Dil.	Stock	DF	Dil.	Stock	DF
8 IX	310	1.05	0.0034	295	1.1	0.0037	300	1.1	0.0037	295	1.1	0.0037	310	1.1	0.0036
9 IX	265	1.1	0.0042	280	1.1	0.0039	290	1.1	0.0038	295	1.1	0.0037	300	1.1	0.0037
	295	1.35	0.0046	290	1.45	0.0050	295	1.40	0.0047	300	1.25	0.0042	300	1.40	0.0047
	295	1.35	0.0046	290	1.45	0.0050	290	1.40	0.0048	295	1.20	0.0041	300	1.30	0.0043
10 IX	275	1.33	0.0048	300	1.36	0.0045	290	1.42	0.0049	300	1.38	0.0046	300	1.30	0.0043
	285	1.3	0.0046	300	1.3	0.0043	280	1.35	0.0048	300	1.35	0.0045	300	1.3	0.0043
11 IX	285	1.33	0.0047	300	1.33	0.0044	280	1.43	0.0051	285	1.4	0.0049	300	1.33	0.0044
	285	1.33	0.0047	295	1.33	0.0045	280	1.43	0.0051	280	1.4	0.0050	290	1.33	0.0046
	265	1.33	0.0050	275	1.37	0.0050	260	1.37	0.0053	275	1.37	0.0049	275	1.37	0.0050
12 IX	260	1.33	0.0051	280	1.33	0.0047	270	1.43	0.0053	270	1.41	0.0052	275	1.33	0.0048
	276	1.3	0.0047	290	1.3	0.0045	285	1.4	0.0049	285	1.4	0.0050	290	1.3	0.0045
13 IX	245	1.25	0.0051	255	1.25	0.0049	245	1.25	0.0051	245	1.25	0.0051	250	1.25	0.0050
	275	1.25	0.0045	275	1.25	0.0045	250	1.20	0.0048	275	1.25	0.0045	275	1.22	0.0044
	300	1.25	0.0042	305	1.25	0.0041	290	1.25	0.0052	300	1.22	0.0041	300	1.25	0.0042
14 IX	315	1.35	0.0043	315	1.35	0.0043	295	1.35	0.0046	300	1.4	0.0047	310	1.3	0.0042
	325	1.15	0.0035	325	1.15	0.0035	305	1.15	0.0038	310	1.15	0.0037	325	1.15	0.0035
	325	1.15	0.0035	325	1.15	0.0035	325	1.15	0.0035	305	1.15	0.0038	—	—	—
15 IX	315	1.1	0.0035	315	1.15	0.0037	300	1.15	0.0038	300	1.1	0.0037	—	—	—
	315	1.1	0.0035	315	1.15	0.0037	295	1.15	0.0039	300	1.1	0.0037	—	—	—
	315	1.3	0.0041	310	1.3	0.0042	285	1.3	0.0046	--	--	--	—	—	—
16 IX	315	1.1	0.0035	315	1.1	0.0035	300	1.1	0.0037	--	--	--	—	—	—
	300	1.1	0.0037	315	1.1	0.0035	300	1.1	0.0037	--	--	--	—	—	—
	285	1.1	0.0039	315	1.2	0.0038	300	1.2	0.0040	--	--	--	—	—	—
17 IX	305	1.2	0.0039	305	1.13	0.0037	285	1.2	0.0042	--	--	--	—	—	—
	300	1.15	0.0038	305	1.15	0.0038	290	1.15	0.0040	--	--	--	—	—	—
18 IX	270	1.1	0.0041	285	1.1	0.0039	260	1.1	0.0042	--	--	--	—	—	—
	290	1.1	0.0038	300	1.1	0.0037	285	1.1	0.0039	--	--	--	—	—	—
	275	1.1	0.0040	280	1.1	0.0039	275	1.1	0.0040	--	--	--	—	—	—
19 IX	285	1.1	0.0039	280	1.1	0.0039	280	1.1	0.0039	--	--	--	—	—	—
	285	1.1	0.0039	280	1.1	0.0039	--	--	--	--	--	--	—	—	—
20 IX	280	1.1	0.0039	280	1.1	0.0039	--	--	--	--	--	--	—	—	—
	280	1.1	0.0039	275	1.1	0.0040	--	--	--	--	--	--	—	—	—
	280	1.1	0.0039	270	1.1	0.0041	--	--	--	--	--	--	—	—	—
21 IX	295	0.9	0.0031	290	1.1	0.0038	--	--	--	--	--	--	—	—	—
	290	1.15	0.0040	290	1.15	0.0040	--	--	--	--	--	--	—	—	—
22 IX	285	1.0	0.0035	300	1.1	0.0037	--	--	--	--	--	--	—	—	—
	280	1.25	0.0045	290	1.25	0.0043	--	--	--	--	--	--	—	—	—
	280	1.25	0.0045	280	1.25	0.0045	--	--	--	--	--	--	—	—	—
23 IX	280	1.25	0.0045	285	1.25	0.0044	--	--	--	--	--	--	—	—	—
	295	1.25	0.0043	280	1.25	0.0045	--	--	--	--	--	--	—	—	—
	305	1.15	0.0038	295	1.25	0.0042	--	--	--	--	--	--	—	—	—
24 IX	250	1.25	0.0050	260	1.25	0.0048	--	--	--	--	--	--	—	—	—
	255	1.1	0.0043	280	1.1	0.0039	--	--	--	--	--	--	—	—	—
25 IX	275	1.25	0.0045	280	1.25	0.0045	--	--	--	--	--	--	—	—	—
	290	1.25	0.0043	280	1.25	0.0045	--	--	--	--	--	--	—	—	—
	290	1.2	0.0041	278	1.2	0.0043	--	--	--	--	--	--	—	—	—
$\bar{x}$	287.1	1.17	0.0042	294.1	1.21	0.0041	285.7	1.23	0.0044	290.5	1.26	0.0043	293.8	1.27	0.0043
S	18.2	0.16	0.0005	21.4	1.04	0.0004	16.9	0.13	0.0006	15.5	0.12	0.0006	17.8	0.09	0.0004
cv	6.3	13.8	12.0	7.3	8.5	10.5	5.9	10.5	14.0	5.3	9.7	12.7	6.1	7.0	10.3

dilution factors (stock flow rate/diluent flow rate) calculated from these data were 0.0042 ± 0.0005, 0.0041 ± 0.0004, 0.0044 ± 0.0006, 0.0043 ± 0.0006, and 0.0043 ± 0.0004. The desired dilution rate was 0.0033. The coefficient of variation ranged from 10.3 to 14.0%. These results are representative of all experiments.

During the early portion of the study, the fail-safe features were not included in the system. Nevertheless, difficulties in maintaining consistent dilution factors were not encountered during experiments except when power failures occurred or after storms which caused high sediment loads in the incoming estuarine water.

This basic dilutor system could be used for all tests with various demands for total flow and concentrations of CIO simply by changing the diluent flow rate and the stock concentrations.

The chlorine stock solutions were prepared by dissolving $Ca(OCl)_2$ in deionized water. The chlorine demand of the deionized water used was usually negligible; however, some experiments had to be eliminated from consideration because stock concentrations declined drastically. Because of this problem, stock solutions were analyzed daily for chlorine concentration by titration with thiosulfate to a starch end-point. Five stock solutions were used to provide the desired concentration series in the test tanks. Since the seawater diluent had a chlorine demand, the relationship between tank concentration and stock concentration at specified flow rates was determined empirically in order to be able to select the appropriate stock concentration to produce any desired CIO residual level. Minor adjustments could be made at the beginning of any given experiment to compensate for variation in demand by adjusting the toxicant flow rate.

Test Chambers

Two-liter plastic aquaria were used for tests with decapod eggs and larvae and *Mulinia* larvae (Fig. 2). Each aquarium (28.5 x 13.4 x 15.0 cm) had a "tidal siphon" drain to produce a fluctuating water level in the test chamber. At an inflow rate of 250 ml/min, the "tidal period" was approximately 7 min.

The test animals were placed in a basket constructed from PVC pipe with a nylon mesh screen glued to the bottom. The basket was suspended at a fixed level in the aquarium. The change in water level in the tank served to flush the basket rapidly enough to ensure equal total chlorine-induced oxidant concentrations inside and outside the baskets. Maximum water volume in the culture baskets was ca. 500 ml.

The screen used for tests with *Mulinia* larvae had a mesh size of 26 μm while that used for decapod eggs and larvae was 274 μm. These

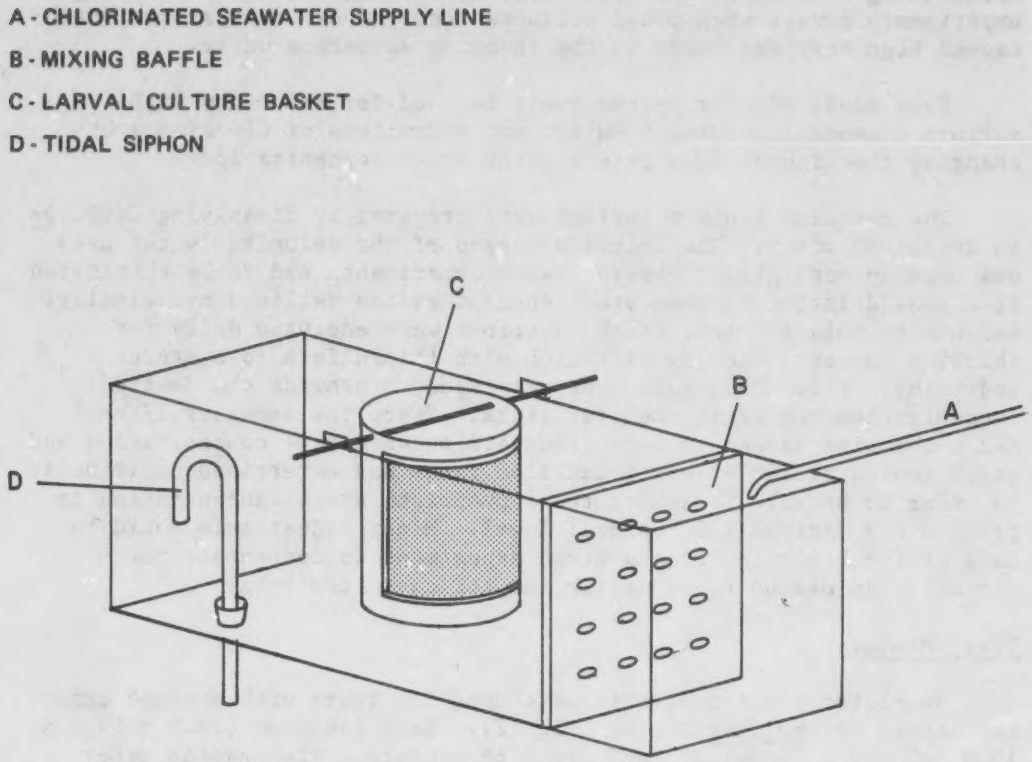
A - CHLORINATED SEAWATER SUPPLY LINE
B - MIXING BAFFLE
C - LARVAL CULTURE BASKET
D - TIDAL SIPHON
C
B
A
D

Figure 2. Test chamber for egg and larval tests.

sizes were selected as the largest size which would retain 100% of the animals introduced or, in the case of crab larvae, the size which would retain Artemia nauplii added as food.

The screen mesh tended to become fouled with aggregations of particles which passed through the diluent filtration systems. The accumulations promoted bacterial and fungal growths, especially in controls and low chlorine doses, with reduced survival of the test species. This could be alleviated by daily rinsing of the baskets in a concentrated chlorine solution followed by thiosulfate to remove residual chlorine while the test animals were removed for counts of survivors. Control survival then equalled or exceeded survival at the lowest test concentration(s).

Chemical Analyses

Various parameters were measured three times daily at approximately 0800, 1200, and 1600 hr during all tests. Temperature and dissolved oxygen were measured in the test tanks with a YSI Model 51A oxygen meter fitted with a BOD oxygen probe; pH was measured with a Perkin Elmer Model 28C pH meter. Salinity of samples from the header tanks was determined with a Beckman Induction Salinometer Model RS-7B.

Total chlorine-induced oxidant concentrations were measured by amperometric titration with phenylarsine oxide (PAO) after iodination at pH 4. A Sargent-Welch Model P Amperometric Titrator was used. A Fisher Recordall strip chart recorder was used to amplify the signal, thereby improving recognition of the titration end point. The electrode system consisted of a rotating platinum-mercury electrode against a calomel. With this system, the minimum detection level was 0.11 μeq/l (0.004 mg Cl_2/l), using 0.000564 N PAO and a 50 ml sample size. Sensitivity could be increased by using a platinum against platinum electrode combination, an electrode with a larger platinum surface area (such as a platinum-mercury hook-type electrode) and/or a polarograph (Andrews and Glass, 1974). However, the instrumentation used was adequate for these tests.

All PAO solutions used were standardized against 0.0025 N $KH(IO_3)_2$. The measured normality of PAO was used in all calculations of CIO concentrations. CIO concentrations are expressed as μeq/l (mg/l Cl_2). 1.1 μeq = 0.04 mg/l Cl_2.

Carpenter et al. (1977) have reported that this method systematically reads less CIO than is actually present. They reported that results were improved somewhat by buffering samples to pH 2. Back titration yielded the most accurate results. Their paper did not appear until the present project was nearing completion, so no change was made in analytical procedures.

General Culture Methods

Ovigerous Panopeus herbstii were collected exclusively on beaches around Gloucester Pt., whereas ovigerous female hermit crabs Pagurus longicarpus were collected either at Gloucester Pt. or from Cedar Island near Wachapreague, Va.

Hatching of eggs from females collected locally was accomplished by placing one or more ovigers in a small aquarium receiving a slow flow of filtered estuarine water. The overflow from the hatching aquarium was passed through a screened basket immersed in estuarine water. As eggs hatched, larvae were carried out of the tank and concentrated in the screened basket. In this way, predation by adults on the newly hatched larvae was reduced or eliminated.

Ovigerous crabs from Wachapreague (salinity ca. 30 °/oo) with eggs nearly ready to hatch were held in standing water of 30 °/oo until hatching, and the larvae were acclimated to York River salinities (18-26 °/oo). Ovigerous crabs with eggs in an early developmental stage were first acclimated to York River salinities, and hatching was carried out as with females from the local population.

Mulinia lateralis adults were obtained from the VIMS field station at Wachapreague. At the main laboratory at Gloucester Pt., the water in which the clams were maintained was adjusted to the ambient salinity of ca. 20 °/oo over a 2-3 day period, after which the clams were maintained in flowing water to which an algal mixture was added either once daily or continuously. When larvae were needed for experiments, spawning was induced by thermal shock and addition of stripped sperm. Eggs were used in experiments immediately after fertilization. Methods used for spawning induction, fertilization, and larval handling were as described by Loosanoff and Davis (1963) and Calabrese (1969a, 1969b, 1970). Static control cultures grown in parallel to most chlorine tests also utilized the methods in those papers. Food was not provided, since the larvae would only begin to feed toward the end of the test period.

Test Protocols

Decapod Egg Development--

Eggs at an early stage of development (only yolk evident on gross examination) were stripped from the pleopods of an ovigerous female with forceps. The egg clumps were teased apart to yield single eggs or small clumps which could be accurately counted. Eggs from single females were divided into groups of approximately 100 and distributed over the series of concentrations to be tested. Each day, the eggs were removed from the culture baskets and examined for mortalities, signs of egg deterioration, and epizootic infestation. Periodically, the eggs were staged to evaluate development. Hatching was recorded as it occurred and larvae were examined grossly for morphological anomalies.

Decapod Larval Tests--

Newly hatched stage I zoeae were placed in culture baskets, 40 zoeae per basket, and exposed to a logarithmic dose series and a diluent control. Newly hatched *Artemia* nauplii were added as food. At daily intervals, larvae were removed from the baskets and examined for mortality. The mortality end point was taken to be lack of heart beat. After enumeration and cleaning of the baskets, larvae were returned to the test chambers with fresh *Artemia* nauplii.

Staging of larvae was accomplished periodically during long experiments. While daily staging would have been desirable, it was decided not to do this because of the time required and the potential for damaging larvae in the process. Development to the megalopal and juvenile stages, readily recognizable without aid of a microscope, was recorded for all larvae surviving to this stage. Most intact dead larvae were staged according to descriptions of Costlow and Bookhout (1961) and Roberts (1969, 1970).

Mulinia Larval Tests--

Two test designs were used with *Mulinia* larvae. In the first design, larvae were exposed to a series of CIO concentrations in the flowing water test system for 48 hr. Larvae were in the trochophore stage at the start of the exposure (about 6 hr after fertilization). At the end of the test, larvae were in the straight hinge stage. Food was not provided as the early larval stages do not eat. After 24 hr, larvae were removed from the baskets briefly so that the baskets could be cleaned. No counts were made at this time, but larvae were examined for development. After a further 24-hr exposure, larvae were removed from the baskets and three quantitative samples counted to estimate the numbers live and dead. Dead larvae in a 1 ml sample placed on a Sedgwick-Rafter slide were first counted visually at 100 X magnification. The sample was then treated with 5% formalin to kill all larvae, and a second count was made.

To reduce the problem of loss of larvae during exposure, a second test protocol was used. In this design, *Mulinia* trochophores were exposed to chlorinated seawater in the flow-through system with flow directed into the culture baskets for 2 hr. Larvae were then removed and counted. Larvae were tabulated as live if any ciliary activity could be detected; otherwise they were recorded as dead. Each population was then cultured in static systems for a variable period and fed with an algal mixture containing equal cell numbers of *Pyramomonas virginica*, *Chlorella* sp. and *Pseudoisochrysis paradoxa*.

Larval population sizes were estimated at 24 hr after exposure and at 48 hr intervals thereafter.

Data Analysis

Mean CIO concentrations for the treatment in each experiment were calculated along with standard deviations and coefficients of variation

$$[\frac{\text{std. dev.}}{\text{mean}} \times 100].$$

The calculated mean CIO was used in subsequent manipulations to derive LC50 values. The data were examined for indications of excessive mortality following major deviations in dose from the mean.

Two methods were used to calculate LC50 values for decapod larvae. Since control survival in larval experiments was less than 100%,using the best available culture method, the data for survival were corrected for control deaths by application of Abbott's Formula (Finney, 1971) for larval experiments 1-6 and 9. In two experiments noted later, control survival was markedly less than that for animals exposed to the lowest dose. In those cases, all data was corrected to assumed 100% survival for animals at the lowest test dose. In all other tests survival at the lowest dose was comparable to control survival. The corrected survival percentages were plotted against log time in hours to determine the LT50. Log LT50 values were then plotted against log mean CIO concentrations to produce a toxicity curve from which the LC50 values for various time intervals could be derived. In cases where control survival was lower than that for the lowest CIO dose, LT50 values were generally similar to those for the next to lowest dose. The second method of calculating LC50 values, used in experiments 7 and 8, involved plotting corrected survival rates (derived as above) against log CIO concentration for each desired time interval and reading the LC50 values from the curves. This method was used in these cases because of the limited number of points available for generation of the toxicity curve. Data for experiment 9 were analyzed by both methods with close agreement in derived LC50 values.

LC50 values for preliminary acute tests with adults of three species of crabs were derived by the second method outlined above but without correction for control mortalities since few or none were observed.

In the *Mulinia* embryo tests, recovery of the larvae introduced was at best less than 100%. Survival percentages were calculated as the volume adjusted number alive at each dose divided by the volume adjusted total number of larvae removed after 48 hr (i.e. number alive divided by number alive plus number dead). The percentages were then plotted against log CIO concentration to derive the LC50 values.

Percent recoveries of larvae were calculated as total number of larvae recovered divided by number of larvae introduced.

RESULTS

Decapod Egg Test

A marginally successful egg test was accomplished only after the method for cleaning culture baskets was developed. Earlier tests yielded data which could not be interpreted because control eggs were badly infected with bacteria and fungi, resulting in inadequate survival and development.

Five measured CIO levels were tested: 1.33 (0.047), 2.23 (0.079), 5.78 (0.205), 25.83 (0.916), and 109.98 µeq/l (3.90 mg/l) (Table 2 and Fig. 3). There was a slight trend for measured CIO residuals to decrease during the test. On two occasions, high CIO levels were observed for short periods, once on the fourth day of the test and again on the ninth day. The cause of the high level on the fourth day, apparent only at the four higher test doses, is unknown. The second anomaly on the ninth day occurred after a 5-hr power outage during the night. This experiment was performed prior to the installation of the fail-safe controls to turn off the toxicant delivery pump. The system was readjusted in the morning about 3 hr after the power failure.

Approximately 100 eggs of Panopeus herbstii in an early stage of development were introduced into each test concentration. Counts of live and dead eggs were made at irregular intervals for 15 days. Eggs were considered to be dead when they became uniformly pale purple (yolk color) without distinct yolk globules, the embryo became opaque and a hyaline space appeared between the egg membrane and the embryo.

Live eggs on day 15 were well eyed and deemed ready to hatch. Chlorine dosing was stopped at this time so that zoeae, as they hatched, would not be exposed to water containing chlorine-induced oxidants. Hatching actually occurred on days 16-18. On day 19, the number of live and dead zoeae was determined to evaluate survival of larvae hatched from chlorine-exposed eggs. The results are summarized in Table 3.

Maximum percent hatch occurred at 1.33 µeq/l (0.047 mg/l) CIO with slightly lower hatch observed at 0.0 µeq/l CIO. No hatch occurred above 2.23 µeq/l (0.079 mg/l) CIO. All eggs exposed to 109.98 µeq/l (3.90 mg/l) CIO were deteriorating by day 5, while those at 25.83 µeq/l (0.916 mg/l) CIO were deteriorating by day 8. Twenty-nine percent of the eggs at 5.78 µeq/l (0.205 mg/l) CIO were alive on day 15, as indicated by presence of a heart beat; none hatched.

TABLE 2. SUMMARY OF CHLORINE-INDUCED OXIDANT CONCENTRATIONS AND WATER QUALITY DATA MEASURED DURING DECAPOD EGG EXPOSURE TEST.

		Control	A	B	C	D	E
Applied dose	µeq/l	0.000	4.29	10.04	22.11	46.81	121.0
	mg/l	0.000	0.152	0.356	0.784	1.660	4.292
Mean residual	µeq/l	0.000	1.33	2.23	5.78	25.83	109.98
	mg/l	0.000	0.047	0.079	0.205	0.916	3.90
Standard deviation	µeq/l	---	0.28	0.48	1.88	9.76	16.95
	mg/l	---	0.010	0.017	0.049	0.346	0.601
Coefficient variation		---	21.3	22.2	24.1	37.7	15.4
Number of samples		45	45	45	45	45	14
Temperature	(°C)	20.3	20.3	20.3	20.3	20.3	20.3
Salinity	(°/oo)	17.8	17.8	17.8	17.8	17.8	17.8
pH		7.85	7.93	7.93	7.94	7.88	7.99
Dissolved oxygen	(mg/l)	8.11	8.09	8.08	8.11	8.08	8.12

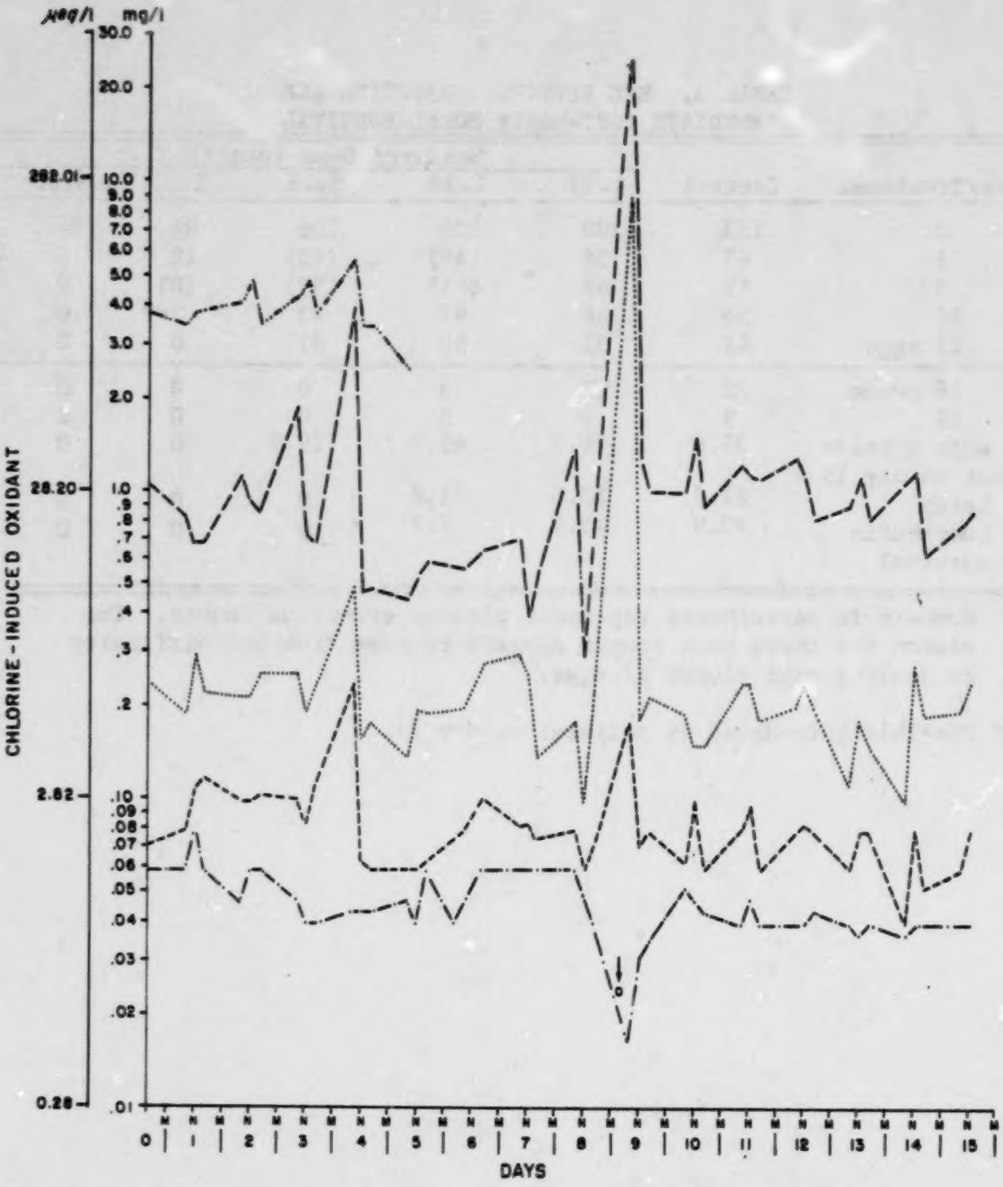

Figure 3. ClO concentrations measured during decapod egg test.

TABLE 3. EGG SURVIVAL, HATCHING AND IMMEDIATE POST-HATCH ZOEAL SURVIVAL

		Measured Dose (μeq/l)				
Day/Treatment	Control	1.33	2.23	5.78	25.83	109.98
0	103	108	110	106	94	94
5	65	56	(46)*	(43)	10	0
8	59	42	(61)	(39)	(0)	0
11	50	46	67	43	2**	0
15 eggs	41	31	50	31	0	0
18 zoeae	22	27	13	0	0	0
19	9	7	3	0	0	0
% eggs c̲ heart beat on day 15	39.8	28.7	45.5	29.2	0	0
% hatch	21.3	25.0	11.8	0	0	0
% post-hatch survival	40.9	25.9	7.7	0	0	0

* Numbers in parenthesis represent clearly erroneous counts. The reason for these poor counts appears to stem from the difficulty in dealing with clumps of eggs.

** Possibly introduced by accident on day 11.

These data suggest that development of the eggs can proceed, albeit with reduced survival, at CIO concentrations up to about 5.78 µeq/l (0.205 mg/l). Hatching can occur at CIO levels up to 2.23 µeq/l (0.079 mg/l) although fewer eggs hatched at 2.23 µeq/l (0.079 mg/l) than at lower doses. Usually the larvae were stage 1 zoeae at hatching; however, one egg at 1.33 µeq/l (0.047 mg/l) hatched as a prezoea on day 15, but did not survive. Hatching success at 1.33 µeq/l (0.047 mg/l) CIO was about equal to that of the control, though both exhibited poor hatchability. However, survival of larvae hatched from eggs exposed to 1.33 µeq/l (0.047 mg/l) was markedly less than that for control larvae.

There are several possible reasons for poor development and hatching of control eggs. First, the eggs used in this experiment were already slightly infested with epizootics, predominantly two species of stalked ciliated protozoans. The effect of such slight infestations is not known, although heavy infestations usually result in poor hatches. High doses of chlorine killed the protozoans, but low doses (1.33 and 2.23 µeq/l) did not. Second, water flow around the eggs in this apparatus was probably less than that provided to eggs attached to female crabs or those cultured on a shaker table. Reduced availability of oxygen at the egg surface could have reduced hatching success. Thirdly, oxygen stress may have occurred during the 5 hr power failure when water flow was interrupted. The excessive residuals produced by the power failure do not appear to have had an immediate effect, since excessive egg mortalities were not observed after this event; however, this does not rule out a chronic effect.

Larval Development Exposures

The effects of chlorine on decapod larvae were assessed with larvae from two species, the estuarine mud crab, *Panopeus herbstii*, and the hermit crab, *Pagurus longicarpus*. Most of the experiments with these animals were performed during the development of the fail-safe features of the diluter or prior to development of an adequate filtration system to provide the desired quantities of sea water during storm periods when sediment loads are extremely high. Hence most experiments do not cover the entire period of larval development.

The experiments presented here are those from which data unaffected by exposure system break-down could be collected for 48 hr or longer.

Panopeus herbstii--

Six acute toxicity tests with *Panopeus herbstii* larvae were performed, varying in duration from two to eleven days. The measured chlorine-induced oxidant (CIO) concentrations in each exposure level are summarized for each experiment in tabular form along with mean temperature, salinity, pH, and dissolved oxygen (Table 4). In general, the coefficient of variation S/X x 100 was between 20 and 50%. The variations are believed to result from variable chlorine demand of the diluent water which was beyond experimental control.

Daily survival rates are shown in Table 5. Despite the variations in measured CIO concentrations, the survivorship curves do not exhibit marked changes in slope corresponding to brief high CIO concentrations; rather, the survivorship curves are essentially linear over the periods of chlorine-induced mortality.

In three experiments, survival of control larvae was less than that for one or more doses during part or all of an experiment. This result was correlated with the aggregation of detrital material on the screen baskets in the control tank and was either not observed or less extensive at the test doses. Bacteria and fungi were associated with this debris. Dissolved oxygen levels were not affected. Presumably the control deaths resulted from bacterial and/or fungal invasion of the larvae. Further support for this interpretation is provided by the fact that the control animal mortality could be reduced by exposing the larval baskets to chlorine followed by thiosulfate while larvae were removed for counting.

The LC50 (lethal concentration for 50% of the population) was derived from toxicity curves of log LT50 (lethal time for 50% of the population corrected for control mortality) against log CIO concentration for 24, 48, and 96 hr (Table 6). In experiments 1, 2, 5, and 6, the 24-hr LC50 ranged from 19.74-28.20 μeq/l (0.7 to 1.0 mg/l), and 48-hr LC50 from 3.67 to 11.56 μeq/l (0.13 to 0.41 mg/l) and the 96-hr LC50 from 1.13 to 3.38 μeq/l (0.04 to 0.12 mg/l). In experiment 3 the LC50's were markedly lower, about 0.28 times the mean for experiments 1, 2, 5, and 6. LC50's for experiment 4 could not be set; the 24-hr LC50 was greater than 32.43 μeq/l (1.15 mg/l) while the 48-hr LC50 was between 12.69 and 32.43 μeq/l (0.45 and 1.15 mg/l). Experiment 3 was performed in October, experiment 4 in early June, i.e. at the end and the beginning of the normal spawning period, respectively. Eggs during these periods are generally more heavily infested with epizootics, which may affect the viability of the resultant larvae, making them less tolerant of pollutants.

Pagurus longicarpus--

Three experiments with *Pagurus longicarpus* larvae yielded useful information. The experiments were of 7, 9, and 21 days duration, the last experiment covering the entire larval period to the first juvenile stage.

The chlorine-induced oxidant concentrations measured during each experiment are summarized in Table 7. The variability at every dose

TABLE 4. SUMMARY OF CHLORINE-INDUCED OXIDANT CONCENTRATIONS AND WATER QUALITY DATA MEASURED DURING *Panopeus herbstii* ZOEAL EXPOSURE

Experiment 1

Treatment	Control	A	B	C	D	E
Applied dose µeq/1 (mg/1)	0.0	14.66 (0.52)	22.00 (0.78)	30.74 (1.09)	45.40 (1.61)	80.37 (2.85)
Mean CIO residual	0.0	7.33 (0.26)	11.56 (0.41)	13.25 (0.47)	17.77 (0.63)	22.56 (0.80)
Stand. deviation	—	2.82 (0.10)	2.54 (0.09)	1.97 (0.07)	2.26 (0.08)	1.41 (0.53)
Coeff. variation	—	38	22	14	13	6
No. of samples	—	4	4	4	4	4
Temperature (°C)	25.2*	25.2*	25.2*	25.2*	25.2*	25.2*
Salinity (°/oo)	18.793	—	—	—	—	—
pH**	—	—	—	—	—	—
Dissolved O_2 (mg/1)	7.7	7.7	7.8	7.9	7.7	7.7

* These measurements were taken one day only.
** pH not taken.

Experiment 2

Treatment	Control	A	B	C	D	E
Applied dose* µeq/1 (mg/1)	0.0	2.26 (0.08)	8.74 (0.31)	20.30 (0.72)	43.71 (1.55)	87.42 (3.10)
Mean CIO residual	0.0	1.13 (0.04)	3.10 (0.11)	6.49 (0.23)	10.72 (0.38)	19.18 (0.68)
Stand. deviation	—	0.28 (0.01)	1.13 (0.04)	2.54 (0.09)	3.67 (0.13)	9.31 (0.33)
Coeff. variation	—	25	36	39	34	49
No. of samples	—	8	8	8	8	8
Temperature (°C)	26.0	26.0	26.0	26.0	26.0	26.0
Salinity (°/oo)	18.353**	—	—	—	—	—
pH***	—	—	—	—	—	—
Dissolved O_2 (mg/1)	6.35	6.35	6.35	6.35	6.35	6.35

* based on measurements from one day.
** taken first day only.
*** pH not taken.

(continued)

Experiment 3

TABLE 4 (continued)

Treatment	Control	A	B	C	D	E
Applied Dose* µeq/1 (mg/1)	0.0	1.41 (0.05)	5.08 (0.18)	14.95 (0.53)	24.25 (0.86)	47.10 (1.67)
Mean CIO residual	0.0	0.85 (0.03)	2.82 (0.10)	5.36 (0.19)	12.13 (0.43)	19.18 (0.68)
Stand. deviation	--	0.56 (0.02)	1.69 (0.06)	1.97 (0.07)	4.23 (0.15)	11.28 (0.40)
Coeff. variation	--	66	60	37	35	59
No. of samples	--	9	6	6	6	7
Temperature (°C)	26.0**	26.0	26.0	26.0	26.0	26.0
Salinity (^{o}/oo)	17.45	17.45	17.45	17.45	17.45	17.45
pH	--	--	--	--	--	--
Dissolved O_2 (mg/1)**	7.0	6.9	6.9	7.0	7.0	7.0

* based on measurements from only first day.
** taken one day only.

Experiment 4

Treatment	Control	A	B	C	D	E
Applied dose µeq/1 (mg/1)	0.0	8.45 (0.30)	12.97 (0.46)	22.28 (0.79)	35.25 (1.25)	61.20 (2.17)
Mean CIO residual	0.0	2.26 (0.08)	4.23 (0.15)	6.77 (0.24)	12.69 (0.45)	32.43 (1.15)
Stand. deviation	--	0.56 (0.02)	0.85 (0.03)	1.41 (0.05)	3.38 (0.12)	9.87 (0.35)
Coeff. variation	--	25	20	21	27	30
No. of samples	--	12	12	11	11	7
Temperature (°C)	23.66	23.66	23.66	23.66	23.66	23.66
Salinity (^{o}/oo)	17.77	17.77	17.77	17.77	17.77	17.77
pH	7.83	7.70	7.83	7.83	7.83	7.80
Dissolved O_2 (mg/1)	8.06	7.97	8.00	8.07	7.97	8.03

(continued)

TABLE 4 (continued)

Experiment 5

Treatment	Control	A	B	C	D	E
Applied dose µeq/1 (mg/1)	0.0	11.00 (0.39)	17.20 (0.61)	30.17 (1.07)	55.27 (1.96)	92.22 (3.27)
Mean CIO residual	0.0	1.13 (0.04)	1.69 (0.06)	8.74 (0.31)	14.95 (0.53)	35.53 (1.26)
Stand. deviation	--	0.28 (0.01)	0.56 (0.02)	7.05 (0.25)	5.92 (0.22)	18.05 (0.64)
Coeff. variation	--	25	33	81	40	51
No. of samples	--	9	8	7	7	2
Temperature (°C)	26.58	26.58	26.58	26.58	26.58	26.58
Salinity (°/oo)	20.71	20.71	20.71	20.71	20.71	20.71
pH	7.71	7.75	7.76	7.76	7.78	7.79*
Dissolved O_2 (mg/1)	6.67	6.62	6.68	6.68	6.70	6.90*

* only one measurement taken.

Experiment 6

Treatment	Control	A	B	C	D	E
Applied dose µeq/1 (mg/1)	0.0	8.46 (0.30)	15.23 (0.54)	23.69 (0.84)	31.30 (1.11)	60.07 (2.13)
Mean CIO residual	0.0	0.85 (0.03)	1.97 (0.07)	4.51 (0.16)	8.74 (0.31)	24.82 (0.88)
Stand. deviation	--	0.28 (0.01)	0.56 (0.02)	1.13 (0.04)	4.51 (0.16)	10.72 (0.38)
Coeff. variation	--	33	28	25	52	43
No. of samples	29	29	29	29	19	5
Temperature (°C)	26.96	26.96	26.96	26.96	26.96	26.96
Salinity (°/oo)	20.03	20.03	20.03	20.03	20.03	20.03
pH	7.81	7.84	7.85	7.88	7.83	7.70*
Dissolved O_2 (mg/1)	6.16	6.30	6.28	6.36	6.45	6.40*

* only one measurement taken.

TABLE 5. SURVIVAL RECORDS FOR *Panopeus herbstii* ZOEAE FOR EACH ClO EXPOSURE TEST

Experiment 1

			Measured Dose (μeq/1)									
	Control		7.33		11.56		13.25		17.77		22.56	
Day	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	40	100	40	100	40	100	40	100	40	100	40	100
1	32	80	23	57.5	31	77.5	20	50	17	42.5	7	17.5
2	26	65	12	30	13	32.5	9	22.5	0	0	0	0

Experiment 2

			Measured Dose (μeq/1)									
	Control		1.13		3.10		6.49		10.72		19.18	
Day	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	37	100	33	100	34	100	30	100	35	100	30	100
1	27	73	26	78.8	30	88.2	23	76.7	27	77.1	1	3.3
2	18	49	26	78.8	25	73.5	19	63.3	25	71.4	0	0
3	16	43.2	21	63.6	2	5.9	0	0	0	0	0	0
4	10	27.0	17	51.5	0	0	0	0	0	0	0	0
5	6	16.2	15	45.5	0	0	0	0	0	0	0	0

Experiment 3

			Measured Dose (μeq/1)									
	Control		0.85		2.82		5.36		12.13		19.18	
Day	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	40	100	40	100	40	100	40	100	40	100	40	100
1	34	85	33	82.5	38	95	19	47.5	0	0	0	0
2	33	82.5	24	60	0	0	0	0	0	0	0	0
3	29	72.5	17	42.5	0	0	0	0	0	0	0	0
4	1	2.5	15	37.5	0	0	0	0	0	0	0	0

(continued)

TABLE 5 (continued)

Experiment 4

Day	Control		Measured Dose (μeq/1)									
			2.26		4.23		6.77		12.69		32.43	
	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	20	100	20	100	20	100	20	100	20	100	20	100
1	18	90	19	95	19	95	17	85	17	85	18	90
2	18	90	18	90	17	85	17	85	17	85	0	0
3	18	90	17	85	15	75	16	80	13	65	0	0

Experiment 5

Day	Control		Measured Dose (μeq/1)									
			1.13		1.69		8.74		14.95		35.53	
	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	20	100	20	100	20	100	20	100	20	100	20	100
1	14	70	18	90	17	85	18	90	13	65	0	0
2	14	70	14	70	12	60	13	65	0	0	0	0
3	13	65	10	50	5	2.5	0	0	0	0	0	0
4	12	60	8	40	0	0	0	0	0	0	0	0
5	12	60	0	0	0	0	0	0	0	0	0	0

Experiment 6

TABLE 5 (continued)

Day	Control		Measured Dose (µeq/1)									
			0.85		1.97		4.51		8.74		24.82	
	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	20	100	20	100	20	100	20	100	20	100	20	100
1	14	70	12	60	15	75	13	65	12	60	0	0
2	13	65	11	55	14	70	10	50	6	30	0	0
3	13	65	11	55	13	65	5	25	0	0	0	0
4	11	55	11	55	12	60	1	5	0	0	0	0
5	8	40	9	45	11	55	0	0	0	0	0	0
6	6	30	9	45	11	55	0	0	0	0	0	0
7	6	30	9	45	4	20	0	0	0	0	0	0
8	4	20	9	45	4	20	0	0	0	0	0	0
9	3	15	9	45	4	20	0	0	0	0	0	0
10	2	10	9	45	3	15	0	0	0	0	0	0

TABLE 6. LETHAL CONCENTRATIONS OF CHLORINE-INDUCED OXIDANTS FOR *Panopeus herbstii* LARVAE

Exp't. No.	Duration of exp't.		LC50			Month test performed
			24 hr	48 hr	96 hr	
1	48 hr	μeq/l	19.74	11.56	--	August
		mg/l	0.70	0.41	--	
2*	144 hr	μeq/l	~ 19.74	8.18	22.20	September
		mg/l	~ 0.70	0.29	0.10	
3	120 hr	μeq/l	~ 6.77	1.69	~ 0.56	late September
		mg/l	~ 0.24	0.06	~ 0.02	
4	96 hr	μeq/l	>32.43	12.69<X<<32.43	--	June
		mg/l	>1.15	0.45<X<< 1.15	--	
5	144 hr	μeq/l	28.20	3.67	1.13	July
		mg/l	1.0	0.13	0.04	
6*	264 hr	μeq/l	25.38-28.20	7.61	3.38	July-August
		mg/l	0.9-1.0	0.27	0.12	

* Based on raw data, LT50 for controls much less than LT50 for lowest dose. Survival rates corrected by using lowest dose as 100%.

TABLE 7. SUMMARY OF CHLORINE-INDUCED OXIDANT CONCENTRATIONS AND WATER QUALITY DATA MEASURED DURING *Pagurus longicarpus* ZOEAL EXPOSURES

Experiment 7

Treatment	Control	A	B	C	D	E
Applied dose μeq/1 (mg/1)	0.0	2.82 (0.10)	3.67 (0.13)	11.84 (0.42)	21.71 (0.77)	33.56 (1.19)
Mean CIO residual	0.0	2.54 (0.09)	2.82 (0.10)	7.05 (0.25)	18.89 (0.67)	36.10 (1.28)
Stand. deviation	—	1.97 (0.07)	1.41 (0.05)	4.51 (0.16)	15.51 (0.55)	1.92 (0.68)
Coeff. variation	—	78	50	64	82	5
No. of samples	—	21	16	16	13	13
Temperature (°C)	26.93	—	—	—	—	—
Salinity (°/oo)	18.86	—	—	—	—	—
pH	7.89	7.83	7.87	7.83	7.90	7.98
Dissolved O_2 (mg/1)	6.94	6.91	8.01	6.96	7.24	7.23

Experiment 8

Treatment	Control	A	B	C	D	E
Applied dose μeq/1 (mg/1)	0.0	16.64 (0.59)	31.30 (1.11)	40.61 (1.44)	70.22 (2.49)	120.98 (4.29)
Mean CIO residual	0.0	0.85 (0.03)	1.97 (0.07)	2.82 (0.10)	7.05 (0.25)	7.61 (0.27)
Stand. deviation	—	0.28 (0.01)	0.56 (0.02)	0.56 (0.02)	1.41 (0.05)	0.85 (0.03)
Coeff. variation	—	33	28	20	20	11
No. of samples	—	22	22	22	13	11
Temperature (°C)	27.63	—	—	—	—	—
Salinity (°/oo)	20.96	—	—	—	—	—
pH	7.87	7.91	7.94	7.94	7.90	7.93
Dissolved O_2 (mg/1)	6.90	6.90	6.93	6.90	7.10	7.05

(continued)

Experiment 9

TABLE 7 (continued)

Treatment	Control	A	B	C	D	E
Applied dose µeq/l (mg/l)	0.0	3.38 (0.12)	7.05 (0.25)	10.72 (0.38)	25.10 (0.89)	38.07 (1.35)
Mean CIO residual	0.0	0.56 (0.02)	1.41 (0.05)	2.54 (0.09)	5.08 (0.18)	10.15 (0.36)
Stand. deviation	--	0.28 (0.01)	0.56 (0.02)	0.85 (0.03)	1.13 (0.04)	1.41 (0.05)
Coeff. variation	--	50	40	33	22	14
No. of samples	--	47	47	31	18	15
Temperature (°C)	23.4	--	--	--	--	--
Salinity (°/oo)	21.21	--	--	--	--	--
pH	7.81	7.82	7.82	7.84	7.80	7.76
Dissolved O_2 (mg/l)	7.02	6.98	6.97	6.96	6.84	6.82

level in experiment 7 was very high with coefficients of variation from 53-83%. In the other two experiments, the coefficients of variation were less than 40%.

The mortality of control animals was relatively uniform during each experiment. In experiments 7 and 9, control survivorship was quite similar to survivorship at the lowest dose (Table 8). The daily point estimates of the survivorship of animals at the lowest dose generally fell within the 95% confidence interval for the survivorship of the control animals. In experiment 8, survival of animals in the lowest dose was unusually low at 192-hr exposure and thereafter, while control survivorship was similar to that in experiments 7 and 9. Survival of static control cultured animals was markedly less than that for control animals in experiments 8 and 9, suggesting that the flow-through culture system was adequate for culture of these animals.

The LC50's for experiment 7 and 8 were obtained by plotting corrected per cent survival against log dose; whereas for experiment 9, the LC50's were derived as for Panopeus larvae. The analysis method used in experiment 7 and 8 was necessary because of the limited number of points available to prepare a toxicity curve (log LT50 vs. log dose). (Values for experiment 9 by both methods were in close agreement.) The LC50 values are presented in Table 9.

The lethal concentrations derived from these three experiments for each time interval are generally similar. The 24-hr LC50 is approximately 11.28 µeq/l (0.4 mg/l), the 48-hr LC50 between 4.51 and 8.74 µeq/l (0.16 and 0.31 mg/l), the 96-hr LC50 between 1.69 and 2.82 µeq/l (0.06 and 0.1 mg/l), and the 120-hr LC50 about 1.41 µeq/l (0.05 mg/l).

The survival data for the entire larval period studied in experiment 9 are shown in Fig. 4 (dose relationship seen here is characteristic of all decapod larval tests). In this experiment, all baskets were rinsed with chlorine followed by thiosulfate on a daily basis after removal of larvae. Survival of the control animals throughout the test period did not differ significantly from that of the animals subjected to the lowest CIO dose of 0.56 µeq/l (0.02 mg Cl_2/l) and was significantly better than that from a control group in static culture. Larvae exposed to doses of 1.41 µeq/l (0.05 mg/l) and above exhibited reduced survival relative to the control animals.

In addition to the effect of CIO on survival, there was a marked effect on the rate of development even at the lowest dose where no effect on survival was observed. The molt from zoea I to zoea II was complete after day 4 or 5 in the control, 0.56, 1.41, and 2.54 µeq/l (0.02, 0.05, and 0.09 mg/l) cultures; but no zoea II were observed at 5.08 and 10.15 µeq/l (0.18 and 0.36 mg/l) prior to death. The larvae exposed to 2.54 µeq/l (0.09 mg/l) died in zoea II. Those larvae exposed to 1.41 µeq/l (0.05 mg/l) which reached the megalopal instar

TABLE 8. SURVIVAL RECORDS FOR *Pagurus longicarpus* ZOEAE FOR EACH CIO EXPOSURE TEST

Experiment 7

			Measured Dose (μeq/1)									
	Control		2.54		2.82		7.05		18.89		36.10	
Day	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	20	100	20	100	20	100	20	100	20	100	20	100
1	18	90	18	90	20	100	20	100	0	0	0	0
2	17	85	16	80	17	85	15	75	0	0	0	0
3	10	50	16	80	13	65	12	60	0	0	0	0
4	10	50	16	80	6	30	0	0	0	0	0	0
5	10	50	14	70	0	0	0	0	0	0	0	0
6	10	50	5	25	0	0	0	0	0	0	0	0

Experiment 8

					Measured Dose (μeq/1)									
	Static Control		Control		0.85		1.97		2.82		7.05		7.61	
Day	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	40	100	40	100	40	100	40	100	40	100	40	100	40	100
1	35	87.5	38	95	36	90	37	92.5	39	97.5	39	97.5	32	80
2	23	57.5	37	92.5	35	87.5	32	80	32	80	28	70	7	17.5
3	22	55	34	85	32	80	27	67.5	28	70	17	42.5	0	0
4	13	32.5	34	85	31	77.5	7	17.5	18	45	0	0	0	0
5	8	20	29	72.5	25	62.5	1	2.5	5	12.5	0	0	0	0
6	8	20	29	72.5	1	2.5	1	2.5	1	2.5	0	0	0	0
7	8	20	28	70	1	2.5	1	2.5	1	2.5	0	0	0	0
8	8	20	27	67.5	1	2.5	1	2.5	1	2.5	0	0	0	0

(continued)

Experiment 9

TABLE 8 (continued)

Day	Static Control 1		Static Control 2		Control		Measured Dose (µeq/1)									
							0.56		1.41		2.54		5.08		10.15	
	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S	n	%S
0	40	100	40	100	40	100	40	100	40	100	40	100	40	100	40	100
1	39	97.5	39	97.5	38	95	40	100	40	100	40	100	40	100	39	97.5
2	36	90	38	95	38	95	39	97.5	39	97.5	40	100	38	95	34	85
3	34	85	36	90	38	95	39	97.5	35	87.5	37	92.5	33	82.5	23	57.5
4	32	80	31	77.5	35	87.5	38	95	32	80	31	77.5	18	45	10	25
5	23	57.5	19	47.5	34	85	36	90	29	72.5	21	52.5	7	17.5	0	
6	18	45	12	30	30	75	36	90	28	70	18	45	0			
7	15	37.5	12	30	30	75	35	87.5	24	60	15	37.5	0			
8	15	37.5	11	27.5	27	67.5	34	85	23	57.5	11	27.5				
9	11	27.5	7	17.5	26	65	29	72.5	23	57.5	4	10				
10	9	22.5	7	17.5	20	50	26	65	13	32.5	0					
11	9	22.5	6	15	20	50	23	57.5	11	27.5						
12	7	17.5	6	15	20	50	22	55	9	22.5						
13	5	12.5	6	15	20	50	21	52.5	6	15						
14	5	12.5	6	15	20	50	21	52.5	5	12.5						
15	5	12.5	6	15	20	50	21	52.5	5	12.5						
16					19	47.5	20	50	5	12.5						
17	4	10	3	7.5	16	40	17	42.5	5	12.5						
18	4	10	3	7.5	14	35	17	42.5	4	10						
19	4	10	3	7.5	12	30	9	22.5	4	10						
20	2	5	3	7.5	9	22.5	8	20	4	10						
21					9	22.5	8	20	3	7.5						

TABLE 9. LETHAL CONCENTRATIONS OF CHLORINE-INDUCED OXIDANTS FOR *Pagurus longicarpus* LARVAE

Exp't. No.	Exp'tal Duration		24 hr	48 hr	96 hr	120 hr
7	144 hr	µeq/l	11.56	8.74	2.82	--
		mg/l	0.41	0.31	0.10	--
8	192 hr	µeq/l	(11.84)	7.61	2.82	1.41
		mg/l	(0.42)	0.27	0.10	0.05
9	504 hr	µeq/l	>11.28	4.51	1.69	1.41
		mg/l	> 0.40	0.16	0.06	0.05

( ) = extrapolated to 50% survival; no dose had less than 50% survival during the first 24 hr.

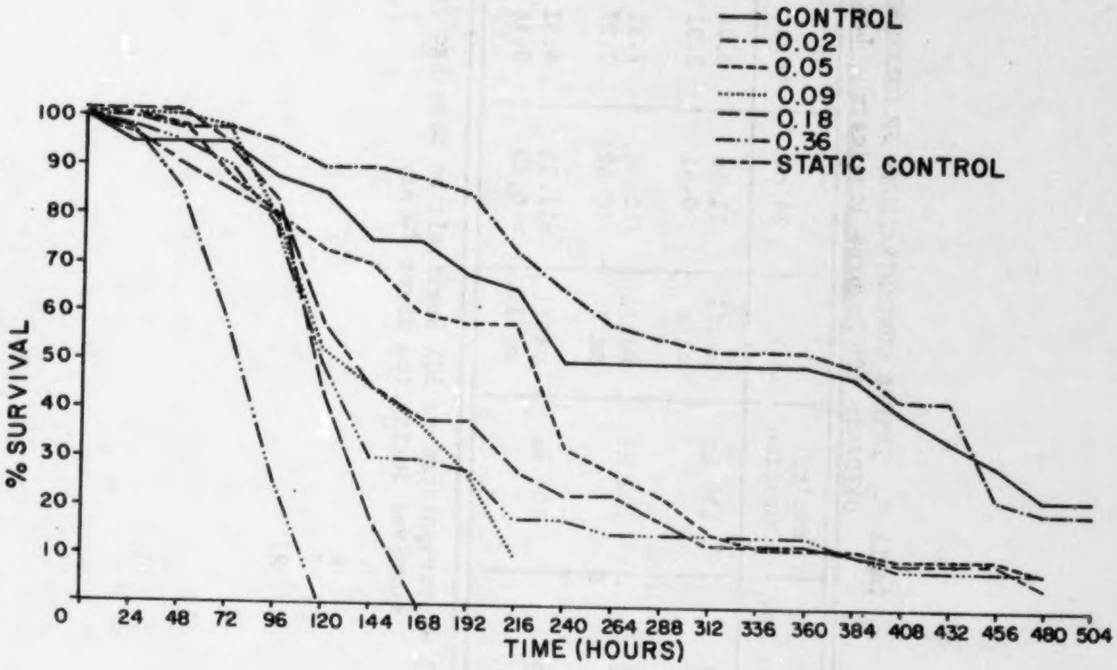

Figure 4. Survivorship curves for *Pagurus longicarpus* zoeae, experiment 9.

(3 animals) did so 5-6 days later than the control animals. The larvae exposed to 0.56 μeq/l (0.02 mg/l) reached the megalopal instar about 1 day later than the controls.

Juvenile Crab Tests

An exploratory experiment with *Panopeus herbstii* juveniles was carried out with feral specimens. The purposes of this experiment were to determine an appropriate dose range for further study and to explore what problems might arise in long term exposures (feeding requirements, cannibalism, disease potential) and what might be done to compensate.

The doses tested ranged from 0.85 to 45.97 μeq/l (0.03 to 1.63 mg/l) (Table 10). Although concentrations were very consistent for periods of a week or more, storms and equipment problems temporarily interrupted diluent flow and prevented maintenance of consistent concentrations for the entire period. Overall, coefficients of variation for all treatments ranged from 29 to 66%. The most variable treatment was the highest dose, which caused complete mortality within 72 hr.

During the first 22 days of the experiment, no substrate was provided to the crabs. Survival rates were high for the first 10 days (80-100%) except for the highest doses (Table 11). Thereafter, survival rates decreased markedly in all treatments including the control. Observation indicated that most if not all deaths resulted from agonistic behavior and cannibalism. Therefore, it was concluded that some type of substrate was necessary to allow crabs to hide, especially during molting.

Two substrates were tested: a layer of sand about 2 cm thick and an assemblage of oyster shells. One tank of each pair at a given dose was provided with the shell substrate (Treatment I), the other with sand substrate (Treatment II). Mortality rates declined following provision of substrates, with some slight advantage seemingly provided by the shell substrate. With the shell substrate, crabs utilized the entire bottom of the aquarium, spending considerable time hiding under shells. Crabs with no substrate or the sand substrate tended to aggregate along the edges of the tank, leading to local high density. With the sand substrate, wandering was reduced as crabs dug into the substrate somewhat, thereby reducing the likelihood of agonistic encounters.

The food provided in this experiment consisted of chopped frozen fish or squid. No attempt was made to quantify food provided. The control crabs and those at low CIO doses fed actively on this food. At higher doses, the crabs were generally less active and were less aggressive in attacking food. No attempt was made to quantify these observations.

TABLE 10. SUMMARY OF CHLORINE-INDUCED OXIDANT CONCENTRATIONS AND WATER QUALITY DATA FOR Panopeus herbstii JUVENILE TEST

		Control		A		B		C		D		E
		I	II	I	II	I	II	I	II	I	II	I
Mean CIO residual	µeq/l	0.00	0.00	0.85	0.85	1.69	1.97	4.79	4.79	7.90	7.61	45.97
	(mg/l)	—	—	(0.03)	(0.03)	(0.06)	(0.07)	(0.17)	(0.17)	(0.28)	(0.27)	(1.63)
Std. dev.	µeq/l	—	—	0.28	0.56	0.56	0.85	2.26	1.41	3.67	3.10	30.46
	(mg/l)	—	—	(0.01)	(0.02)	(0.02)	(0.03)	(0.08)	(0.05)	(0.13)	(0.10)	(1.08)
Coef. var.		—	—	33	66	33	43	47	29	46	41	66
No. samples		—	—	42	42	42	42	42	42	42	42	5
Temperature °C			19.7	—	—	—	—	—	—	—	—	—
Salinity, °/oo			16.8	—	—	—	—	—	—	—	—	—
pH*			7.9	7.9		7.8		7.9		7.9		N.D.
Dissolved O_2, mg/l			7.9	7.9		7.9		7.9		7.9		N.D.

N.D. - no data
* - no data for first 19 days due to instrument malfunction
CIO expressed as µeq/l (mg/l)

TABLE 11. SURVIVAL DATA FOR Panopeus herbstii JUVENILE TEST

			Measured Dose (µeq/l)								
Day	Control		0.85	0.85	1.69	1.97	4.79	4.79	7.90	7.61	45.97
	I	II	I	II	I	II	I	II	I	II	I
0	10	10	10	10	10	10	10	10	10	10	7
1	10	10	10	10	10	10	10	10	10	10	5
2	10	10	10	10	10	10	10	9	10	10	1
3	10	10	10	10	10	10	10	9	10	10	0
4	10	10	10	10	10	9	10	9	10	10	
5	10	10	10	10	10	9	10	9	10	9	
6	10	10	10	10	9	9	10	9	10	9	
7	10	10	10	10	9	9	10	9	10	9	
8	10	10	10	10	9	9	10	9	10	9	
9	10	10	10	10	9	9	10	9	10	9	
10	8	10	10	10	9	9	10	9	10	9	
11	6	10	10	9	9	9	10	9	10	9	
12	6	10	10	9	9	9	10	9	10	9	
13	4	10	10	8	8	9	10	9	9	8	
14	4	10	10	8	8	9	10	9	9	8	
15	4	10	10	8	8	9	10	9	9	8	
16	4	9	10	8	8	9	10	9	8	8	
17	4	9	10	7	8	7	10	8	8	6	
18	4	9	10	7	8	7	10	8	8	5	
19	4	9	10	7	8	7	10	8	8	4	
20	4	9	10	7	8	7	10	8	8	4	
21	4	9	10	6	8	7	10	8	8	4	
22	4	9	10	5	8	7	10	8	8	4	
23	4	9	10	5	8	7	10	8	8	4	
24	3	9	9	5	8	7	10	6	8	3	
25	3	9	9	5	8	7	9	5	8	3	
26	3	9	9	5	8	7	9	4	8	3	
27	3	9	9	5	8	7	9	4	8	3	
28	3	9	9	5	8	7	9	4	8	2	
29	3	8	9	5	8	7	9	4	8	2	
30	3	8	9	5	8	7	9	4	8	2	
31	3	8	9	5	8	7	9	4	8	1	
32	3	8	9	5	8	7	9	4	8	1	
33	3	8	9	4	8	7	9	4	8	1	
34	3	8	9	4	8	7	9	4	8	1	
35	3	8	9	4	8	7	9	4	8	1	
36	3	6	9	3	8	7	9	4	8	1	
37	3	6	9	3	8	7	9	4	8	1	
38	3	6	9	3	8	7	9	4	8	1	
39	3	6	9	3	8	7	9	4	8	1	
40	3	6	8	3	8	7	9	4	8	1	

Deaths at the highest dose level of 45.97 μeq/l (1.63 mg/l) were attributable to the chlorinated seawater. An approximate 96-hr LC50 of 14.10 eq/l (0.50 mg/l) was estimated. No deaths at any other dose were clearly attributable to the chlorine although the exposure continued for 40 days. Juvenile crabs can tolerate exposure to at least 7.90 μeq/l (0.28 mg/l) for extended periods.

Exploratory tests were also conducted with *Pagurus longicarpus* adults and *Callinectes sapidus* juveniles in order to determine the approximate toxic concentrations for each species. The 24-hr, 48-hr and 96-hr LC50's for *Pagurus* adults were estimated to be 16.07, 14.10, and 5.92 μeq/l (0.57, 0.50, and 0.2 mg/l) respectively. The 24-hr, 48-hr, and 96-hr LC50's estimated for *Callinectes* juveniles were 12.41, 11.84, and 9.02 μeq/l (0.44, 0.42, and 0.32 mg/l), respectively. These values must be considered preliminary pending more careful and detailed experiments.

Mulinia lateralis Experiments

Several experiments were performed with *Mulinia lateralis* larvae exposed for the 48 hr immediately following fertilization. The exposure conditions for the larval experiments are summarized in Table 12. In experiment 1, test condition A had a higher mean CIO residual than condition B although the range was essentially the same. At these doses and below, it becomes increasingly difficult to maintain consistent residuals, and analytical error increases greatly. This is obvious in experiment 2 in which the lowest dose was 0.25 μeq/l (0.019 mg/l) with a coefficient of variation of about 100%. The temperature in experiment 1 was low (18°C), but sufficient to allow development to the straight hinge.

Experiment 1 was conducted before institution of the basket cleaning process. Control survival was less than that for the lowest dose as was total percent recovery of the animals introduced (Table 13). Control survival and recovery were also markedly less than the same parameters for a static control culture.

Only the lowest dose tested (0.99 μeq/l or 0.035 mg/l) permitted survival. At 1.04 μeq/l (0.037 mg/l), all dead animals had failed to reach the straight hinge stage. Recovery percentage declined with increasing dose. This has been characteristic of tests with oyster larvae as well. It is not clear whether chlorine simply fails to preserve the larvae, or whether the dead larvae are oxidized by the chlorine.

Poor survival in the control and survival at only one test dose preclude determination of an LC50. It would appear, however, that the LC50 is less than 1.41 μeq/l (0.05 mg/l) and probably greater than 0.85 μeq/l (0.03 mg/l) since survival and recovery of larvae at the lowest dose approximated results from the static culture.

TABLE 12. SUMMARY OF CHLORINE-INDUCED OXIDANT CONCENTRATIONS AND WATER QUALITY DATA MEASURED DURING *Mulinia lateralis* EMBRYO TESTS

Experiment 1

Treatment	Static Control	Control	A	B	C	D
Applied dose µeq/1 (mg/1)	0.0	0.0	5.41 (0.192)	6.66 (0.236)	26.37 (0.935)	30.54 (1.083)
Mean CIO residual	0.0	0.0	1.04 (0.037)	0.99 (0.035)	1.78 (0.063)	2.48 (0.088)
Stand. deviation	--	--	0.71 (0.025)	0.59 (0.021)	0.37 (0.013)	0.31 (0.010)
Coeff. variation	--	--	68	60	21	13
No. of samples	--	--	7	7	7	7
Temperature (°C)	--	18.33	--	--	--	--
Salinity (°/oo)	--	17.96	--	--	--	--
pH	--	7.85	7.86	7.86	7.83	7.83
Dissolved O_2 (mg/1)	--	8.43	8.47	8.40	8.43	8.43

Experiment 2

Treatment	Control	A	B	C	D	E
Applied dose µeq/1 (mg/1)	0.0	1.69 (0.060)	13.87 (0.492)	5.33 (0.189)	9.73 (0.345)	15.81 (0.561)
Mean CIO residual	0.0	0.25 (0.009)	0.76 (0.027)	1.33 (0.047)	2.17 (0.077)	3.86 (0.137)
Stand. deviation	--	0.25 (0.009)	0.23 (0.008)	0.45 (0.016)	0.59 (0.021)	0.82 (0.029)
Coeff. variation	--	100	30	34	27	21
No. of samples	--	7	7	7	7	7
Temperature (°C)	23.5	--	--	--	--	--
Salinity (°/oo)	18.21	--	--	--	--	--
pH	8.00	8.05	8.00	8.00	8.00	8.05
Dissolved O_2 (mg/1)	7.65	7.05	7.70	7.80	7.75	7.75

(continued)

Experiment 3

TABLE 12 (continued)

Treatment	Static Control	Control	A	B	C	D	E
Applied dose µeq/l (mg/l)	0.0	0.0	1.78 (0.063)	2.48 (0.088)	6.26 (0.222)	10.01 (0.355)	16.75 (0.594)
Mean ClO residual	0.0	0.0	0.53 (0.019)	0.96 (0.034)	1.72 (0.061)	3.47 (0.123)	7.33 (0.260)
Stand. deviation	--	--	0.11 (0.004)	0.45 (0.016)	0.51 (0.018)	2.03 (0.072)	0.93 (0.033)
Coeff. variation	--	--	20	46	29	59	13
No. of samples	--	--	6	6	6	6	6
Temperature (°C)	--	28.0	--	--	--	--	--
Salinity (°/oo)	--	20.0	--	--	--	--	--
pH	--	8.27	8.37	8.37	8.30	8.30	8.33
Dissolved O_2 (mg/l)	--	7.25	7.26	7.47	7.30	7.18	7.23

Experiment 4

Treatment	Static Control	Control	A	B	C	D	E
Applied dose µeq/l (mg/l)	0.0	0.0	1.64 (0.058)	2.62 (0.093)	4.94 (0.175)	10.41 (0.369)	18.89 (0.670)
Mean ClO residual	0.0	0.0	0.28 (0.010)	0.53 (0.019)	1.18 (0.042)	2.26 (0.080)	5.64 (0.200)
Stand. deviation	--	--	0.39 (0.014)	0.11 (0.004)	0.31 (0.011)	0.45 (0.016)	0.62 (0.022)
Coeff. variation	--	--	146	22	27	20	11
No. of samples	--	--	6	6	6	6	6
Temperature (°C)	--	25.8	--	--	--	--	--
Salinity (°/oo)	--	22.8	--	--	--	--	--
pH	--	7.67	7.60	7.53	7.60	7.60	7.63
Dissolved O_2 (mg/l)	--	7.19	7.17	7.22	7.20	7.20	7.20

(continued)

Experiment 5

TABLE 12 (continued)

Treatment	Static Control	Control	A		B		C		D		E	
Applied dose µeq/1 (mg/1)	0.0	0.0	1.40	(0.049)	2.78	(0.099)	5.04	(0.179)	9.08	(0.322)	16.79	(0.556)
Mean CIO residual	0.0	0.0	0.45	(0.016)	0.90	(0.032)	1.24	(0.044)	2.40	(0.085)	5.64	(0.200)
Stand. deviation	—	—	0.09	(0.003)	0.09	(0.003)	0.37	(0.013)	0.25	(0.009)	2.23	(0.079)
Coeff. variation	—	—	19		9		28		10		39	
No. of samples	—	—	6		6		6		6		6	
Temperature (°C)	—	21.0	—		—		—		—		—	
Salinity (⁰/oo)	—	15.0	—		—		—		—		—	
pH*	—	—	—		—		—		—		—	
Dissolved O_2*(mg/1)	—	—	—		—		—		—		—	

* No data.

TABLE 13. SURVIVAL, MORTALITY AND RECOVERY RESULTS FOR 48-HR *Mulinia lateralis* EMBRYO EXPOSURE TESTS

Experiment 1

CIO concentration µeq/1	(mg/1)	Initial number	Live @ 48 hr (straight hinge) N	% survival	Dead @ 48 hr (straight hinge) N	(Embryo) N	% recovery
0.00	0.000	6090	3100	89	400	0	57
0.99	0.035	5250	3550	82	800	0	83
1.04	0.037	5670	0	0	0	1267	22
1.78	0.063	4830	0	0	0	417	9
2.48	0.088	4830	0	0	0	100	2
Static Control		3780	2800	92	250	0	81

Experiment 2

CIO concentration µeq/1	(mg/1)	Initial number	Live @ 48 hr (straight hinge) N	% survival	Dead @ 48 hr (straight hinge) N	(Embryo) N	% recovery
0.00	0.000	4074	240	86	40	0	7
0.25	0.009	4912	160	77	47	0	4
0.76	0.027	4340	198	69	90	0	7
1.33	0.047	4774	119	37	203	0	7
2.17	0.077	4466	0	0	299	1131	32
3.86	0.137	4453	0	0	0	210	5

(continued)

TABLE 13 (continued)

Experiment 3

CIO Concentration µeq/1	(mg/1)	Initial Number	Live @ 48 hr N	% Survival	Dead @ 48 hr N	% recovery
0.00	0.000	8165	113	81	27	2
0.53	0.019	7495	644	58	468	15
0.96	0.034	6994	1380	90	160	22
1.72	0.061	10472	287	56	230	5
3.47	0.123	8175	292	55	240	7
7.33	0.260	7228	0	0	0	0
Static Control		8342	4806	95	267	61

Experiment 4

CIO Concentration µeq/1	(mg/1)	Initial Number	Live @ 48 hr N	% Survival	Dead @ 48 hr N	% recovery
0.00	0.000	4464	840	72	322	26
0.28	0.010	4096	208	34	399	15
0.53	0.019	3869	209	42	285	13
1.18	0.042	5320	116	35	213	6
2.26	0.080	3735	0	0	15	0.4
5.64	0.200	4785	0	0	0	0
Static Control		4512	3239	88	430	81

(continued)

Experiment 5

TABLE 13 (continued)

CIO Concentration µeq/l	(mg/l)	Initial Number	Live @ 48 hr N	% Survival	Dead @ 48 hr N	% recovery
0.00	0.000	5205	2728	100	0	52
0.45	0.016	5175	116	100	0	2
0.90	0.032	5236	240	71	100	6
1.24	0.044	5440	0	0	100	2
2.40	0.085	4940	0	0	121	2
5.64	0.200	5553	0	0	234	4
Static Control		5260	4660	89	0	89

Overall recovery of larvae in experiment 2 was poor (4 to 7% in most treatments) because larval baskets with 37 μm mesh were used instead of baskets with 26 μm. Mulinia embryos are larger than 37 μm, but may be deformed to pass through 37 m at the 1-, 2-, and 4-cell stages.

Experiment 2 was carried out after the basket cleaning process was instituted. Control survival exceeded survival at all dose levels (Table 13). The static control was inadvertently discarded before counting. Therefore, we cannot evaluate how much of the observed mortality was attributable to culture conditions versus viability of the larval batch.

Survival was reduced progressively with increasing dose beginning with the lowest dose (0.25 μeq/l or 0.009 mg/l). No embryos survived at 2.17 μeq/l (0.077 mg/l) or above. At 2.17 μeq/l (0.077 mg/l) some embryos developed to the straight hinge stage prior to death, whereas at 3.86 μeq/l (0.137 mg/l) all animals died as embryos. The 48-hr LC50 determined from these data was 1.07 μeq/l (0.038 mg/l) within the range suggested by the first experiment.

Experiments 3, 4, and 5 were conducted with water entering the culture baskets directly rather than using the tidal siphons. Percent recoveries of larvae were again low. The LC50's for these experiments cannot be precisely estimated statistically. The LC50 for experiment 3 appears to be over 2.82 μeq/l (0.1 mg/l). For experiment 4, the LC50 is much lower near 0.28 μeq/l (0.01 mg/l). In experiment 5, the LC50 was about 0.93 μeq/l (0.033 mg/l). The low recovery rates for larvae and the difficulties in maintaining consistent CIO levels over a 48 hr test at these low concentrations do not allow a stronger statement than that the LC50 for 48 hr continuous exposure lies between 0.28 μeq/l and 2.82 μeq/l (0.01 mg/l and 0.1 mg/l).

When larvae were exposed for only 2 hr in the continuous flow system (experiments 6 and 7), larval recoveries were generally better than 50% and often around 100% (Table 14). Survival rates were also high except at CIO levels above 2.82 μeq/l (0.1 mg/l). The 2 hr LC50's for these experiments were 2.06 μeq/l (0.073 mg/l) and 1.97 μeq/l (0.070 mg/l), respectively. The survival curves during the subsequent static culture period were approximately parallel indicating no residual mortality effects after removal from chlorinated water. Larvae did not grow significantly in these 9 day experiments even in control cultures and hence did not metamorphose. This probably reflects the inadequacy of small culture volumes and high larval densities for culture of bivalve larvae (Dupuy et al., 1977; Windsor, 1977).

TABLE 14. SURVIVAL AND RECOVERY RESULTS FOR 2-HR *Mulinia* EMBRYO EXPOSURE TESTS AND SUBSEQUENT STATIC CULTURE

Experiment 5

Time		CIO Concentration µeq/1 (mg/1)						
		0.00	0.25 (0.009)	0.51 (0.018)	0.79 (0.028)	1.52 (0.054)	2.99 (1.00)	Static Control
before exposure	N	9453	9372	9675	10234	10361	10133	9322
after exposure	N	9072	7728	8064	9406	7666	1995	9430
	% S	96	82	83	92	74	20	101
	% R	96	82	83	92	74	52	101
24 hr.	N	6536	6467	7397	8900	*	**	5394
	% S	72	84	92	95	--	--	57
72 hr.	N	4933	2811	6650	6673	--	1603	5647
	% S	54	36	82	71	--	80	60
120 hr.	N	1067	1353	2920	2520	--	1650	2532
	% S	12	18	36	27	--	82	27
168 hr.	N	660	1276	1133	2210	--	550	1325
	% S	7	17	14	23	--	28	14

* This culture was spilled when the 24 hr. count was attempted.

** Culture volume was not recorded so N could not be estimated.

(continued)

Experiment 6

TABLE 14 (continued)

Time		CIO Concentration µeq/1 (mg/1)						
		0.00	0.23 (0.008)	0.59 (0.021)	1.10 (0.039)	1.69 (0.060)	4.23 (0.150)	Static Control
before exposure	N	10368	13321	9635	12352	12413	10346	11936
after exposure	N	11100	7033	11333	6933*	7067*	1233	11867
	% S	107	53	118	56	57	12	107
	% R	107	53	118	90	79	91	107
24 hr.	N	6033	1600	6533	8567	8200	533	9433
	% S	54	23	58	124*	116*	43	79
72 hr.	N	4067	933	3967	6000	6633	433	6767
	% S	37	13	35	87	94	35	57
120 hr.	N	1867	633	1833	1833	2133	500	3700
	% S	17	9	16	26	30	40	31
168 hr.	N	966	100	333	1900	967	567	2900
	% S	9	1	3	27	14	46	24
216 hr.	N	633	367	0	833	833	67	1767
	% S	6	5	0	12	12	5	15

* The number alive was underestimated based on the criteria of ciliary activity. Therefore the percent survivals based on these counts are overestimated. However the scope of the survivorship curves (not shown) was unaffected.

SECTION 5

AVOIDANCE BEHAVIOR EXPERIMENTS

METHODS

Larval Culture

Pagurus longicarpus zoeae for avoidance experiments were obtained from laboratory populations. Adults were obtained from Cedar Island near Wachapreague, Va. or Gloucester Point, Va. The adult crabs were maintained on a sea table receiving a continuous supply of unfiltered or 10 µm filtered estuarine water. A thin layer of subtidal sand was provided as a food-containing substrate. Small pieces of frozen fish were added every other day. Purina Marine Chow was added periodically as a food supplement. A minimum 14-hr light--10 hr dark photoperiod was maintained by means of a timer connected to two 30 W fluorescent tubes (daylight white) located directly above the sea table. The photoperiod was lengthened during summer months by natural lighting from windows adjacent to the sea table. Water temperature was maintained at 20°C or above by passing the incoming water through a thermo-regulated water bath.

Each week the adult populations were examined for ovigerous females. Ovigerous crabs were placed in an aquarium receiving 1 µm filtered estuarine water. The discharge water flowed through a tube into a screened basket (254 µm mesh) immersed in a water bath. Larvae released in the tank were removed with the effluent water and collected in the screened basket containing freshly hatched Artemia nauplii which were replaced daily. The basket was examined daily for newly hatched zoeae which were then placed in 20.3 cm diameter glass bowls until used in experiments.

Larvae were maintained in static cultures according to methods described by Roberts (1969, 1970). The culture medium was 1 µm filtered estuarine water. Newly hatched Artemia nauplii were provided daily as food.

Experimental Apparatus

The apparatus used to evaluate the response of larvae to chlorinated estuarine water was designed specifically to deal with a nonconservative material, chlorine-induced oxidants, and to accommodate decapod larvae which respond primarily in the vertical plane. In principle, the apparatus provides a flowing two-layered water column with chlorine in the upper layer. Larvae are introduced into the lower layer and induced to swim upward by a directed light source. In this way, the expected avoidance response, vertically downward, can be elicited. To maintain consistent ClO levels, each layer is continuously replenished.

The final apparatus design settled on as providing the necessary conditions is shown in Figures 5 and 6. Figure 5 depicts diagrammatically the complete apparatus. Water was supplied from a header tank (a) to a flow splitter tank (b) which in turn supplied the constant level header tanks (c and d). Tanks c and d were placed on laboratory jacks so that flow rates could be finely adjusted by changing their heights relative to the test chambers. Gross flow adjustment was achieved with screw clamps. Tank c supplied water for the upper layers of each test chamber while tank d supplied the lower layers. Inflow rates were measured with flow meters (i). The outflow rates were regulated adjusting the height of tubes attached to the outflow ports (k and p). The flow rates were selected to provide a horizontal velocity of approximately 0.167 cm/sec for each layer which is sufficiently slow to prevent larvae being trapped against the nylon mesh screens. Outflow rates for each layer must equal the inflow rates for that layer in order to maintain a sharp discontinuity. A Vycor heater and temperature probe in tank c with thermostat (j) maintained the temperature slightly above that in tank d (0.5 to 1°C) which helped insure discrete layers in chambers e and f. A mechanical stirrer in tank c (not shown) kept the water temperature homogeneous. In order to maintain the test water temperatures during passage from the header tanks (c and d) to the test chambers, much of the tubing was passed through water jackets (k) receiving water from the same header tanks as the test chambers they supplied. The two test chambers were enclosed in a darkened box. Two 20 W fluorescent lights were located above the test chambers to provide a directional stimulus for the larvae. Two holes were provided in one side of the box to allow visual or photographic observations of the larvae (not shown).

The test chamber (Figure 6) was located inside a water bath to provide thermal insulation. Water entered the water bath through a port (A) at one end and exited through a port (B) at the same end after passing around the test chamber. Water entered the test chamber through two inflow ports (C and D), one for the upper layer and one for the lower layer. Incoming water to the upper layer passed from chamber E through a diffuser plate (F) into a second chamber (G) and thence into the test chamber through a diffuser plate and nylon mesh screen (H, 254 μm mesh size). Incoming water to the lower layer followed a similar path from chamber L, through diffuser plate M into chamber N and then through the diffuser plate and nylon mesh screen (H). The layers flowed out through a nylon mesh screen and diffuser

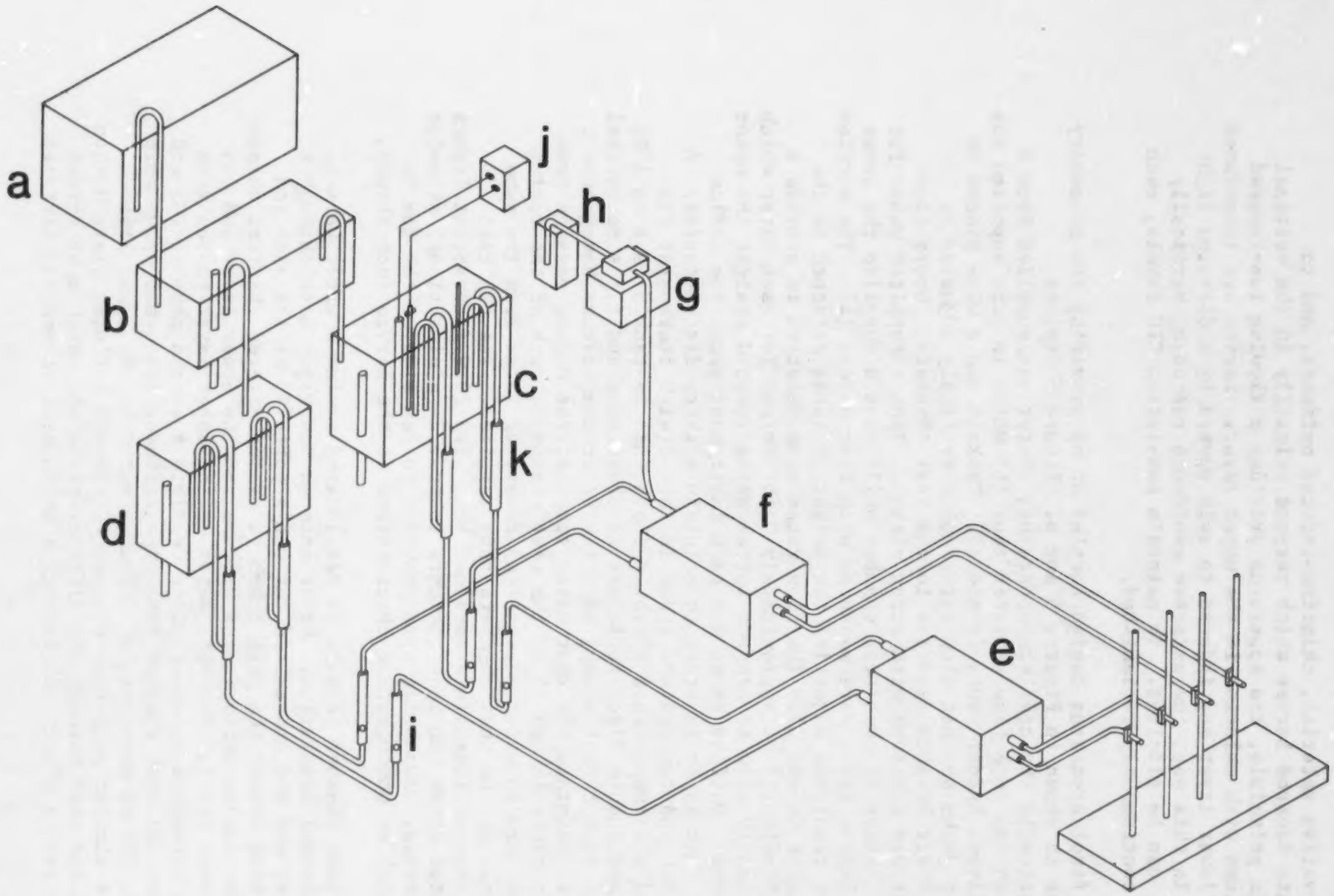
a
b
c
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Figure 5. Diagrammatic representation of complete avoidance test apparatus.

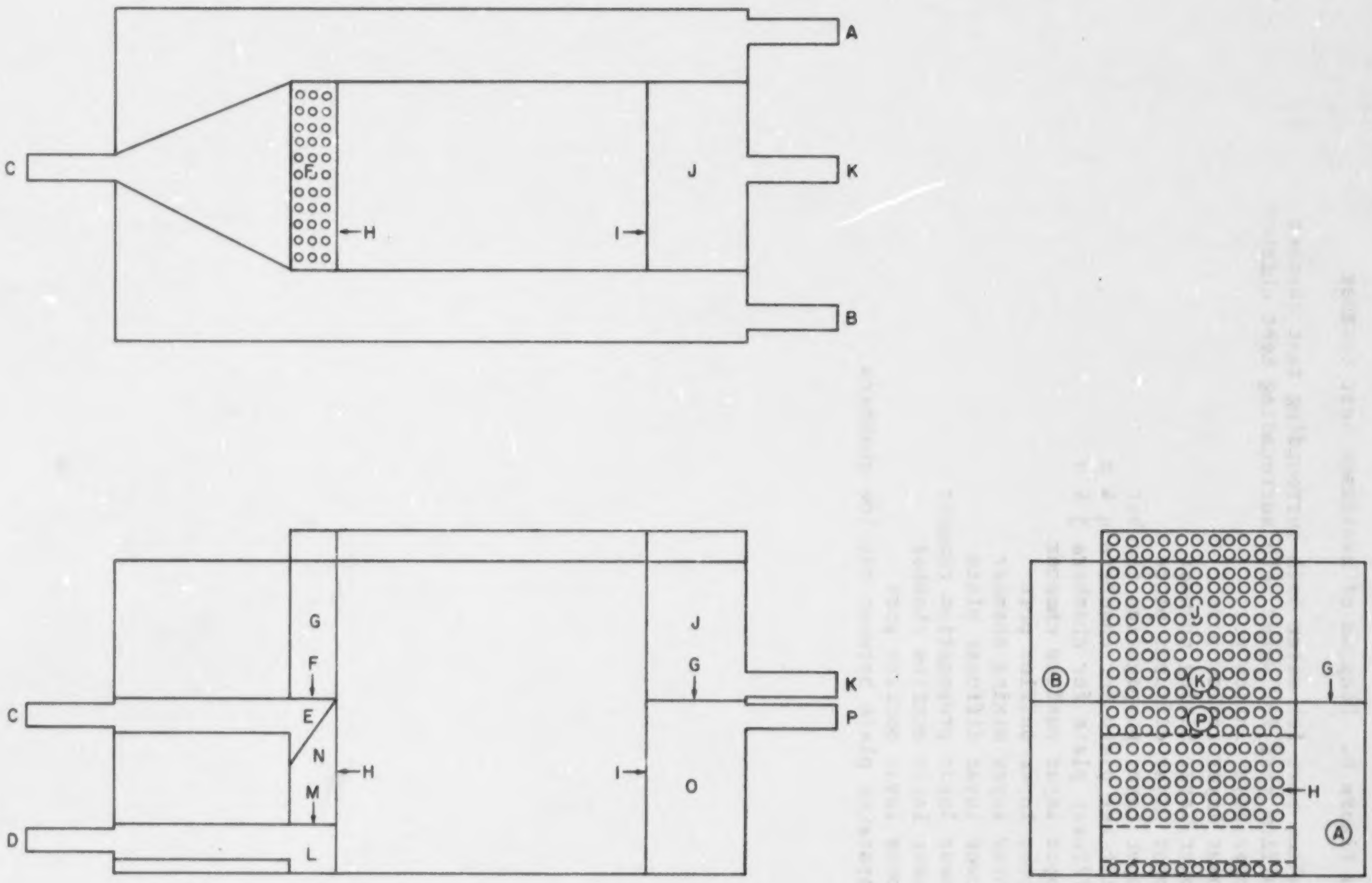
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Figure 6. Diagrammatic representations of avoidance test chamber. Upper view, longitudinal cross section through center, and view from outflow end.

Key to Figure 6. Diagram of avoidance test chamber

A. inflow port for water bath surrounding test chamber
B. outflow port for water bath surrounding test chamber
C. upper layer inflow port
D. lower layer inflow port
E. upper layer mixing chamber
F. upper layer diffuser plate
G. upper layer propagation chamber
H. diffuser plate for chambers g & n
I. diffuser plate for chambers j & o
J. upper layer outflow chamber
K. upper layer outflow port
L. lower layer mixing chamber
M. lower layer diffuser plate
N. lower layer propagation chamber
O. lower layer outflow chamber
P. lower layer outflow port
Q. separator plate between outflow chambers

plate (I) into chambers J (upper layer) and O (lower layer) and out ports K and P respectively.

A peristaltic pump (Fig. 5, g) was used to inject a $Ca(OCl)_2$ solution into the upper layer inflow tube just downstream of the flow meter (i). Injection may be made into either chamber. The resultant ClO concentration in the upper layer was measured amperometrically just before and just after each avoidance test. Larvae were injected into the apparatus through a port located centrally in the bottom of each test chamber.

Dye Studies

In order to determine the discreteness of the two layers, rhodamine B and fluorescene dye were injected into the upper and lower layers, respectively. Dye distribution was observed for periods in excess of one hour.

In a typical dye experiment, the two layers were well delineated over the test period. Under certain conditions, colored water from the upper layer infiltrated the lower layer along a narrow strip immediately adjacent to the side walls. This problem has been overcome by adding holes in the diffusor plate close to the sides of the test chambers which improved flow along the sides of the tank.

Experimental Protocol

For preliminary experiments with chlorine applied to the upper layer, two test chambers were set up and flow rates adjusted. The upper layer of one chamber received the desired concentration of chlorine; the other chamber served as a control. After a period for equilibration, the ClO level of the upper layer was determined.

For each test 20-40 stage I zoeae were introduced into the lower, unchlorinated layers of both chambers with a large-bore syringe. Direct visual observations were made every five minutes with notes on the observable behavior (direction of swimming, telson flip response, passive sinking, etc.).

Preliminary Experiments

Preliminary experiments without chlorine were designed to evaluate three areas of concern: 1) do larvae behave in the same way within the two test chambers, 2) what is the effect of light intensity on the number of larvae exhibiting a positive response, and 3) does salinity affect the number of larvae exhibiting a positive response?

To answer the first question, each chamber was operated without chlorine injection with water of 18‰ salinity. Maximal light intensity of 240 fc was used in one test, 24 fc (achieved by introducing neutral density filters between the light source and test chamber) in a second experiment. Larvae introduced into each chamber were observed for over 1 hr.

The effect of light intensity was evaluated under the same conditions except that 240 fc was applied to one chamber, 24 fc to the other. In a second experiment, the light intensities were switched for the two chambers. Larval responses were observed for over 1 hr.

Salinity effects were observed at 240 fc and 24 fc in separate experiments. The salinities tested were 18 °/oo, at which positive responses were expected to be low, and 24 °/oo, at which positive responses were expected to be high. This salinity range brackets approximately the usual salinity range for York River water entering the laboratory. If the difference in larval response was large, we felt it would be necessary to add a means of adjusting salinity to some uniform level for these tests.

Data Analysis

Photographic observations, as originally planned, were not feasible because of low light intensities. Instead, the number of larvae in each horizontal quarter section of the test chamber was determined by visual observations.

Observations were collected in a time series, and hence any numerical data analysis must consider the time factor in some way. For each observation time, the number of larvae in the upper layer (n_u) and in the lower layer (n_L) were tablulated for each experiment. The proportion of the larvae in the upper layer was then calculated as follows:

$$p = \frac{n_u}{n_u + n_L} \times 100$$

We desired to test the null hypothesis that there was no difference in the level of response during the test period. Therefore we performed a Wilcoxon Sign Rank test (Steele and Torrie, 1960) on the differences between p in the test and control chambers. Although this approach ignores the magnitude of response differences, no truly appropriate statistical procedure was identified for analysing the magnitude of response differences. Those test conditions producing significantly different responses were thus identified, and the directions of the responses were determined by inspection.

RESULTS

Preliminary Experiments

At both light intensities, there was no difference in the number of *P. longicarpus* larvae in the upper layers of the two chambers in any experiment. The level of response was significantly different between replicate experiments at a given light intensity in some cases. This difference between experiments is attributed to the fact that larvae from different females were used. At 240 fc the larvae responded rapidly, whereas at 24 fc the number of larvae responding increased gradually during the first 35 min of the experiment (Fig. 7, 8).

In experiments comparing response to a light intensity of 240 fc versus 24 fc, conflicting results were obtained. In one experiment, the larvae exhibited a significantly greater response at 24 fc than at 240 fc (based on a Wilcoxon Sign Rank Test). In a replicate test, the responses were not significantly different at the 95% level, but again the stronger response was observed at 24 fc (Fig. 9). In the first test, the mean response at 24 fc was 46%, whereas in the second test it was 79%; i.e. the number of positive responses was high in the test showing no significant differences.

Larvae exposed to 240 fc gave a stronger positive response in 18 °/oo water than in 24 °/oo water. In two cases the response was significantly greater throughout the experiment. In a third experiment, there was no significant difference overall, but larvae in 18 °/oo water gave a stronger positive response than those in 24 °/oo water for the first 38 min (Fig. 10). In one experiment, the mean percent responding positively was extremely low (7.1 and 23.9%). Larvae used in this test were 48-hr-old and were probably ready to molt to stage II.

At 24 fc, larval responses as a function of salinity were inconsistent. In two experiments, larvae gave a significantly greater response at 18 °/oo than at 24 °/oo (Fig. 11). In a third experiment, the responses were not significantly different although larvae in 18 °/oo water gave a stronger positive response than those in 24 °/oo water for the first 28 min.

To summarize these experiments, the mean response was determined at each time interval for all tests performed under identical light intensities and salinities despite the high variability in responses between tests. The high variance prevents demonstration of statistically significant differences. Graphically it can be seen that there was no consistent overall response of larvae to light intensity. The larvae at 240 fc showed a higher positive response up to 35 min and thereafter a lower response than the larvae at 24 fc. At 24 °/oo the larval response to 240 fc was generally lower than the response to 24 fc. Larvae tested at 18 °/oo salinity gave greater responses than those tested at 24 °/oo at both light intensities. Thus, the levels of response were not as greatly affected by light intensity as by salinity (Table 15, Fig. 12).

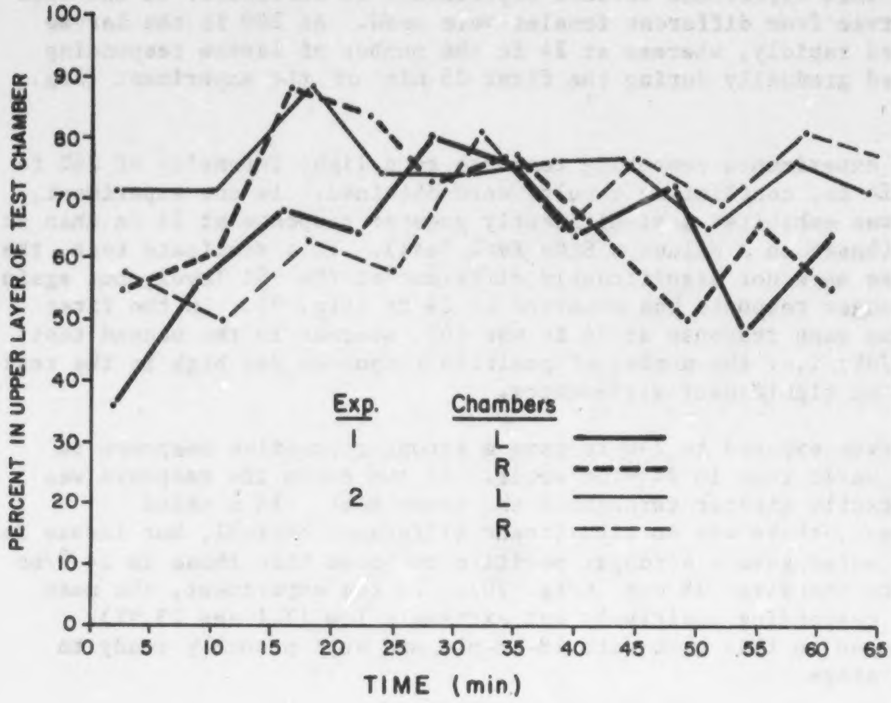

Figure 7. Percent of Pagurus longicarpus stage I larvae in the upper half of the test chambers versus time (min) when exposed to 240 fc in 18 ⁰/oo salinity water.

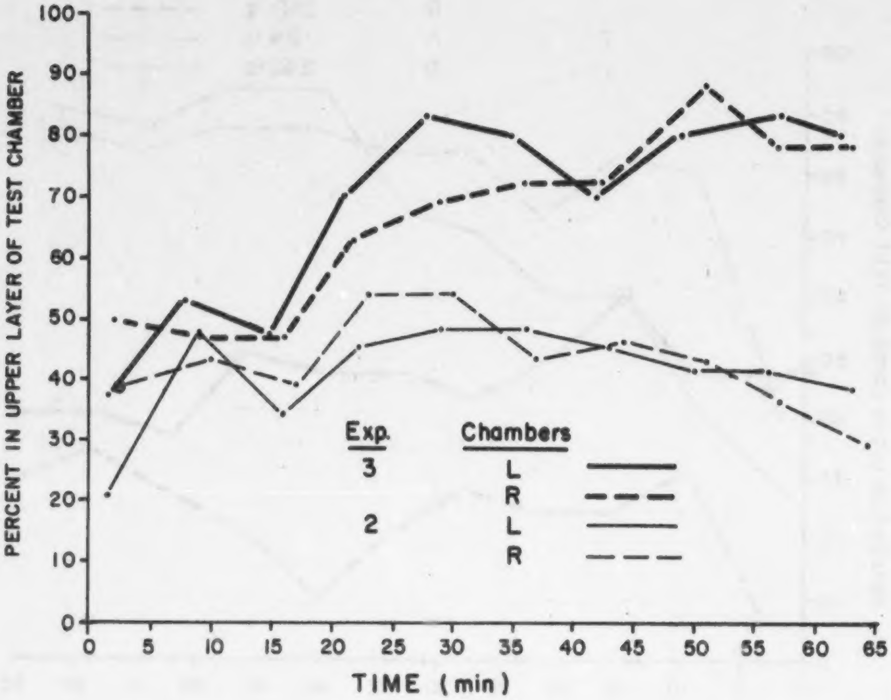

Figure 8. Percent of Pagurus longicarpus stage I larvae in the upper half of the test chambers versus time (min) when exposed to 24 fc in 18 ⁰/oo salinity water.

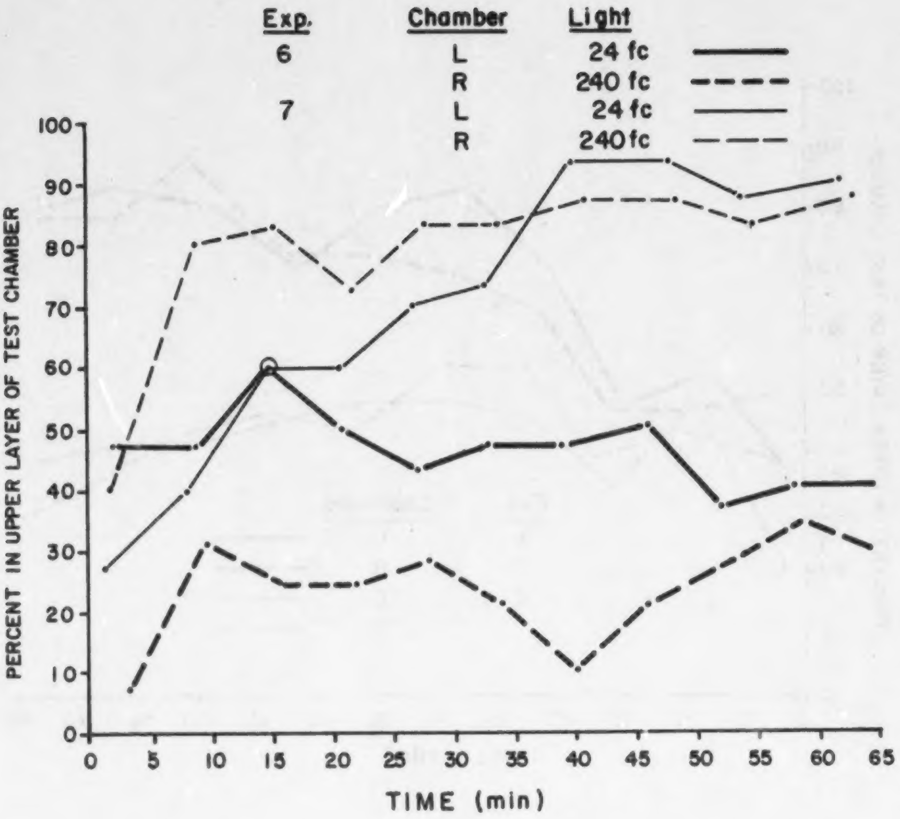

Figure 9. Percent of Pagurus longicarpus stage I larvae in the upper half of the test chambers versus time (min) when exposed to 240 fc and 24 fc in 18 °/oo salinity water.

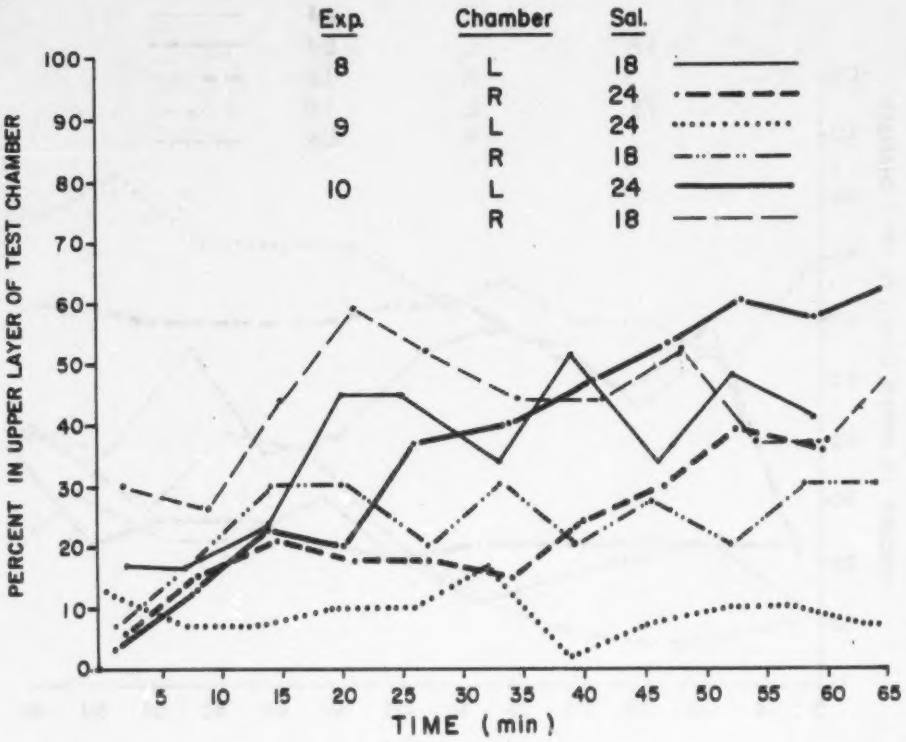

Figure 10. Percent of Pagurus longicarpus stage I larvae in the upper half of the test chambers versus time (min) when exposed to 18 ^{o}/oo and 24 ^{o}/oo at 240 fc.

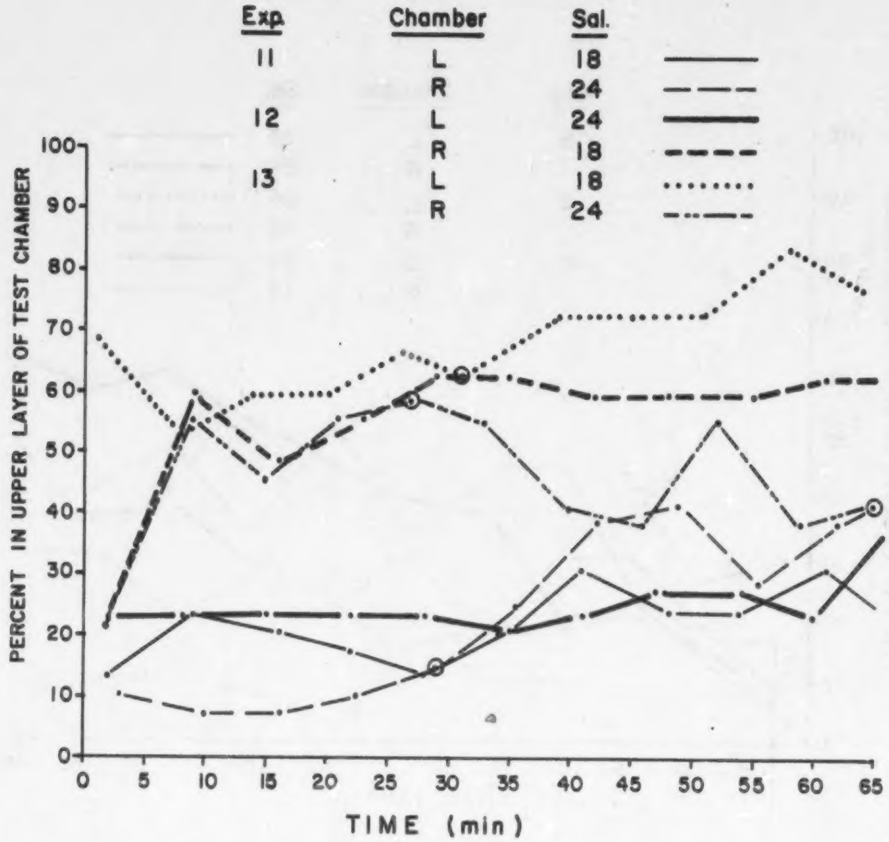

Figure 11. Percent of Pagurus longicarpus stage I larvae in the upper half of the test chambers versus time (min) when exposed to 18 ^{o}/oo and 24 ^{o}/oo at 24 fc.

TABLE 15. SUMMARY OF PERCENT PHOTOPOSITIVE RESPONSES FOR STAGE 1 *Pagurus longicarpus* LARVAE AT 5-MIN INTERVALS ARRANGED ACCORDING TO TREATMENT. (DATA FOR REPLICATE TESTS AVERAGED)

Salinity ($^{o}/oo$)	18				24			
Light Intensity (fc)	24		240		24		240	
Observation time (min)	$\overline{x}$	SD	$\overline{x}$	SD	$\overline{x}$	SD	$\overline{x}$	SD
5	40.4	12.2	34.1	22.5	19.7	9.1	9.3	0.6
10	40.1	10.1	46.9	22.1	27.7	23.7	13.2	5.4
15	46.6	13.1	55.0	24.6	25.0	19.4	17.0	7.8
20	47.5	17.2	58.4	22.0	28.3	22.8	16.0	5.3
25	54.5	18.2	56.7	21.5	30.5	24.0	20.7	12.2
30	56.4	19.5	58.3	23.7	31.2	22.6	23.0	13.1
35	57.6	19.3	57.1	24.5	31.2	16.8	22.7	16.1
40	59.3	20.1	53.9	25.3	32.2	9.6	24.2	21.8
45	59.9	20.9	55.1	23.9	34.2	8.0	28.8	22.0
50	59.5	24.0	53.2	21.3	38.2	11.3	33.5	23.8
55	58.5	24.1	53.6	20.9	34.0	11.4	35.7	24.6
60	58.9	24.1	54.8	21.6	32.2	8.1	34.2	24.5
No. of tests averaged	9		9		3		3	

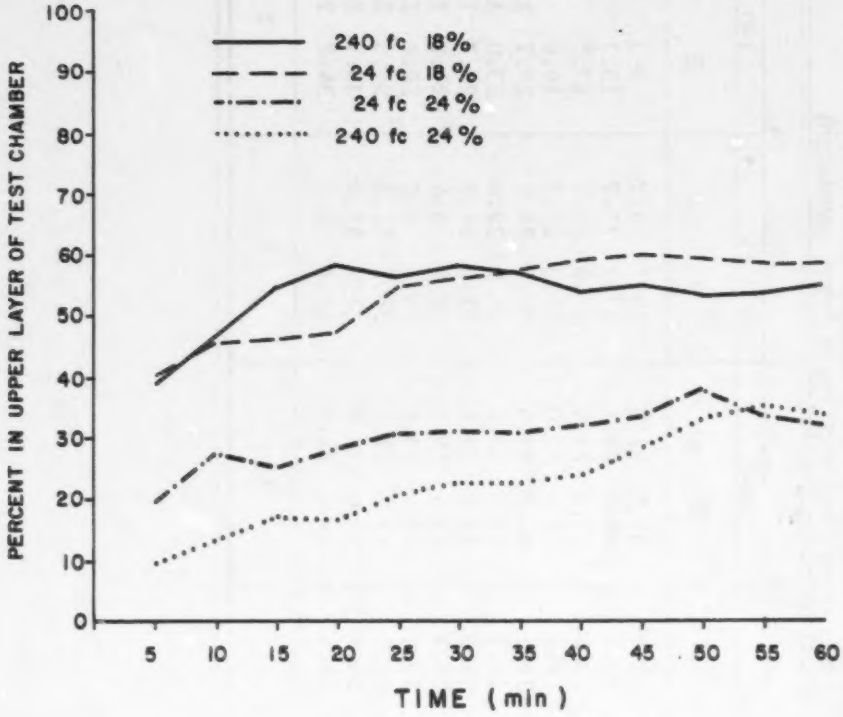

Figure 12. Mean percent of _Pagurus longicarpus_ stage I larvae in the upper half of the test chambers versus time (min) for all experiments at each light intensity-salinity combination.

Responses to Chlorinated Seawater

Larvae of *P. longicarpus* were not available for tests of the effect of a chlorinated seawater layer scheduled to be initiated in January. As the preliminary tests were being completed, the laboratory population ceased reproduction as a result of reduced salinity. Salinity of the incoming water dropped to 13 ^{o}/oo, which is below the level permitting egg development to hatching (Roberts, 1969). At this time of year ovigerous females are not available in the field at any location within the geographic range of the species.

A trial experiment was carried out earlier with *Palaemonetes pugio* larvae. The primary purpose of the test was to test the larval introduction system and the feasibility of photographic observations. Hence no quantitative response data were collected. The test conditons were 240 fc, 20 ^{o}/oo salinity, and 70.5 μeq/l (2.5 mg/l) in the upper layer. Larvae were observed visually to determine the type(s) of responses produced. Three types of larval responses as described by Roberts (1969, 1971; telson flips, downward swimming, and passive sinking), were observed when larvae encountered the chlorinated layer. Larvae in the control chamber entered the upper layer without responding to the two water layers.

These studies will be continued by one of the authors (J.E.I.) as a thesis project.

SECTION 6

SEROLOGICAL EFFECTS

METHODS

Experimental Animals

Adult blue crabs were obtained from two sources. At the beginning of the study crabs were obtained from the Chesapeake Bay potting fishery. The severe crab shortage in Chesapeake Bay during the winter of 1976-77 forced us to import crabs by air from the North Edisto River in South Carolina for several subsequent tests. This source was used until July 1977 when Chesapeake Bay crabs were once again available.

The nature of the crab fisheries in both localities makes it impossible to be precise about the temperature and salinity regimes from which the crabs were taken, but it can be assumed that the Chesapeake Bay crabs came from salinities (20-25 ^{o}/oo) 5-10 ^{o}/oo lower than the North Edisto River crabs (∿30 ^{o}/oo). Local crabs were taken from summer water temperatures (20-30°C) while those from Bears Bluff were collected during early spring and summer (water temperatures ∿15-25°C). No attempt has been made to compare physiological measurements between the two populations, but crabs from only one locality have been used for a given series of tests.

All crabs were held for 7-14 days prior to their use. The initial Chesapeake Bay crabs were held in flowing York River water. The South Carolina and later Chesapeake Bay crabs were held in a recirculating sea water system heated to room temperature (20.5-27°C). The water was continuously filtered to 5-10 µm. Approximately one-third of it was replaced daily with 5 µm filtered York River water (17.4-24.6 ^{o}/oo). All crabs were fed squid daily, and, although their claws were bound to prevent cannibalism, they had no apparent problems in feeding.

Exposure Systems

The basic dilutor system was similar to that described in detail in Section 4.

A crude estimate of ClO LC50 for adult blue crabs was obtained using three doses and a control. The exposure system consisted of four aerated 18-liter (5-gallon) aquaria receiving chlorinated York River water directly from individual mixing boxes. Chlorine stock solutions of various concentrations were pumped into a diluent flow of approximately 300 ml/min. The tanks each held five adult crabs.

The exposure system used for long-term (six-week) serological studies consisted of six tanks, each with eight 3-liter compartments for the crabs. This system was designed to allow exposure of eight physically isolated crabs to each of five exposure levels and one control level without cannibalism. Each tank was supplied through a header box which received chlorinated water from a mixing box. Each animal compartment received water through a calibrated siphon (100 ml/min). Appropriate exposure levels were obtained by pumping stock solutions of varied concentrations into a mixing box with a diluent flow of approximately 800 ml/min.

This 48-compartment dosing system proved too cumbersome to maintain. It was discovered that the crabs were depleting their ambient O_2 supply by up to 25%. Doubling flow rates through the system did not solve the problem, and aeraton of the system was impractical. In addition, the number of crabs available from the system for blood analysis was shown to be too small given the high variances observed for the blood parameters.

These reasons, coupled with our growing suspicions that crab responses were more rapid than previously anticipated, led us to use a system of eight 37-liter (10-gallon) aquaria with higher flow rates (1 liter/min) and vigorous aeration to maintain oxygen levels at near saturation. There was virtually no effect of aeration on the ClO concentrations. The tanks were arranged in two groups of four so that a given test consisted of one dosed group and a control group. Each group of four tanks received water from a single header. Toxicant was mixed as before, except that diluent flow was increased to ca. 4800 ml/min. The flow rates and aeration of this system allowed ten crabs per tank. Coupled with the new experimental design, the sample size at each interval was increased from the four crabs in the previous system to ten crabs.

An additional system was used to determine whether tank size and flow rates might have influenced early tests. The experimental group included one 37-liter (10 gallon) and two 18-liter (5-gallon) tanks. The 37-liter and one 18-liter tank were supplied by a single header with a diluent flow of ca. 4800 ml/min. Tank flows were ca. 1000 ml/min and ca. 500 ml/min, respectively. The remaining 18-liter tank received a flow of ca. 250 ml/min from a separate header receiving diluent at a rate of ca. 1100 ml/min. The control group had similar tanks with similar flows drawn from a single header. In each group, the 37-liter tank contained ten crabs while each 18-liter tank contained five.

Experimental Protocols

The LC50 tests were performed by exposing five adult crabs to each of three doses and a control. Doses were obtained by diluting stock solutions of varying concentrations determined to give the desired CIO levels in the tanks. Stable CIO levels were established before crabs were put into the tanks. CIO concentrations and crab mortalities were monitored twice daily, and flow rates into the tanks were determined daily. Each test was terminated at the end of 96 hr; any remaining crabs were bled.

The protocol for the 48-compartment system called for eight mature female crabs sampled at time zero as a baseline, and a maximum of 24 experimental (dosed) and 24 control (undosed) crabs. In the tests, four experimental and four control crabs were randomly sacrificed for serum analysis at selected intervals until all were utilized or had died. CIO concentrations, mortalities, dissolved oxygen, temperature, and salinity were monitored three times daily. Flow rates and pH were determined once per day.

The protocol for the 37-liter (10-gallon) aquarium system required that ten mature female crabs be sampled at time zero as a baseline. Forty crabs (ten per tank) were dosed with a single CIO level, and 40 more crabs served as controls. At the end of each day of the four day test, ten dosed and ten control crabs, representing the entire contents of randomly selected tanks, were sacrificed for analysis. CIO levels, temperature, salinity, dissolved oxygen, and mortality were measured three times daily; pH and flow rates were recorded once daily.

In the final test using the 18- and 37-liter tanks, ten baseline crabs were sampled at time zero. Twenty crabs (ten per 37-liter tank; five per 18-liter tank) were dosed, matched by twenty controls. Surviving crabs were sampled after three days. CIO levels, temperature, salinity, dissolved oxygen, mortality, pH and flow rates were monitored as before. In addition, NH_3-N levels were determined daily for each tank and for the diluent water by the method of Solorzano (1969).

Methods of Serological Analysis

Six or seven milliliters of blood were usually taken from each crab by withdrawal from the sinus at the base of the fifth pereiopod by means of a plastic syringe with an 18 gauge needle. Samples were placed in capped test tubes in an ice water bath. The samples were allowed to chill for at least 30 minutes, after which the clot in each was broken up and centrifuged out (2000 g for 30 min). The decanted serum was then divided into at least two portions and frozen. Analyses were performed on samples frozen and thawed only once.

Constituent analyses were made using procedures described in detail for blue crab serum by Lynch and Webb (1973a) for TNPS (total ninhydrin positive substances), Lynch and Webb (1973b) for protein, Lynch and Webb (1973c) for glucose and Lynch, Webb and Van Engel (1973) for chloride and osmotic concentration.

Data Analysis

Preliminary 24-, 48- and 96-hr LC50's were determined from a plot of percent survival versus log CIO concentration representing a composite of data from four different tests for lethal limits. Serum constituent values from two of these tests were subjected to one-way analysis of variance.

Statistical analysis of the data from the 48-compartment system/six-week tests was unproductive because of the small sample sizes and the large variances encountered. The overlap between standard errors precluded detection of significant differences.

Time course and concentration effects on serum from the four-day tests were evaluated by factorial analysis. The analysis was performed on individual 2 x 4 factorial tests where the factors were dose (one dose plus control) and time (one, two, three and four days), respectively.

The final-3 day test was evaluated by one-way analysis of variance and a modified Tukey's ω'-procedure (Steel and Torrie, 1960) for effects of tank size-flow-CIO combinations on crab serum constituents. A separate one-way analysis of variance and standard Tukey's ω-procedure were used to determine effects on tank NH_3-N levels.

RESULTS

Lethal Limits

Groups of five mature crabs were exposed to twelve different CIO concentrations ranging from 3.1 to 315.8 μeq/l (0.11 to 11.20 mg/l) and a control for 96 hr at approximately 20°C. Total mortality was observed at 141 μeq/l (5.0 mg/l) after 24 hrs, 71.3 μeq/l (2.53 mg/l) after 48 hrs and 45.1 μeq/l (1.6 mg/l) after 96 hrs. Total survival was observed after 96 hrs at 18.3 μeq/l (0.65 mg/l) and less. The 96-hr LC50 was 28.8 μeq/l (1.02 mg/l); the 48-hr LC50 was 30.2 μeq/l (1.07 mg/l); the 24-hr LC50 was 91.7 μeq/l (3.25 mg/l) (Fig. 13). Mortality estimates from other four day tests were roughly comparable to these data, although direct comparisons are difficult because of differing experimental conditions. Mortality after 96 hr was 0% at 10.2, 11.8, and 14.1 μeq/l (0.36, 0.42 and 0.50 mg/l), 38.3% at 13.3 μeq/l (0.47 mg/l), 30.9% at 13.5 μeq/l (0.48 mg/l) and 24.2% at 17.5 μeq/l (0.62 mg/l). Mortality after 48 hr was 23.4% at 29.3 μeq/l (1.04 mg/l).

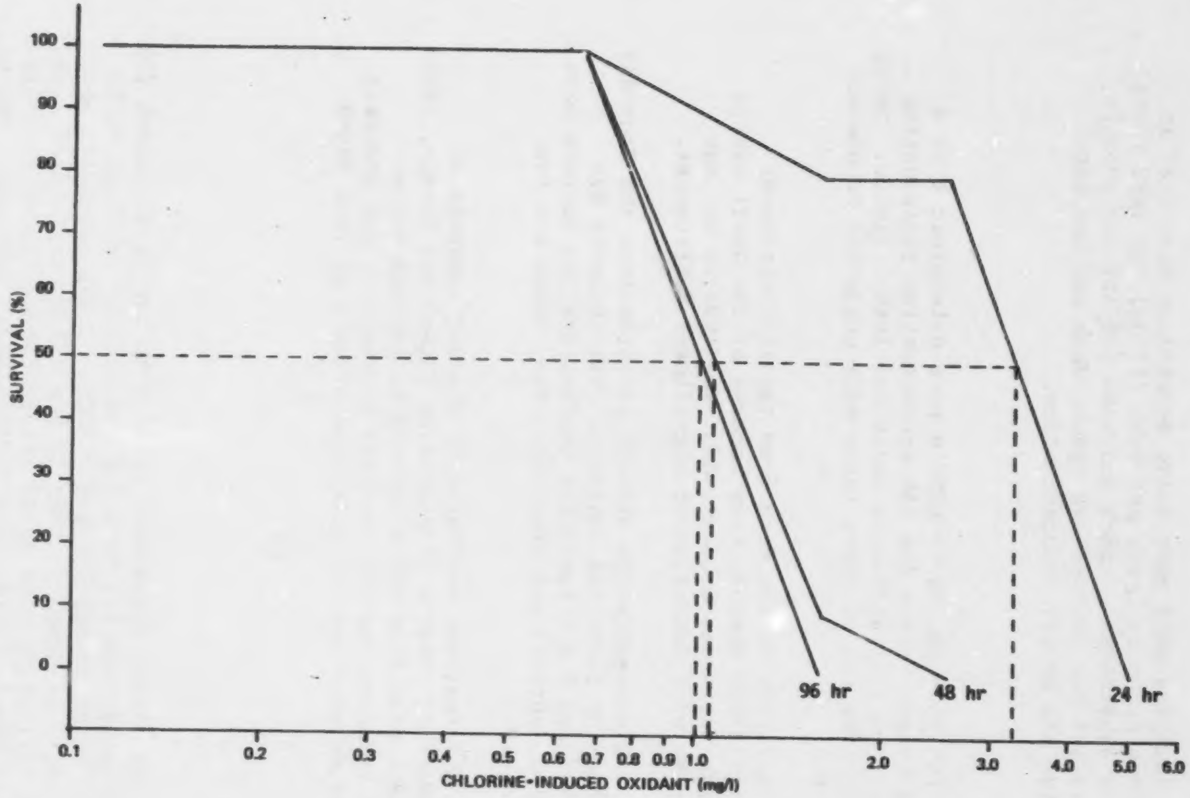

Figure 13. Composite summary of survival data for Callinectes sapidus adults plotted against log ClO concentration. Includes data from 4 experiments. The LC50 values are considered to be preliminary only.

Time Course and Concentration Effects on Serum

Serum from thirteen different experiments was analyzed for glucose (first experiment only), TNPS, protein, chloride and total osmotic concentrations (Table 16). CIO levels and other hydrographic data appear in Table 17. Mean serum constituent values and other pertinent data can be found in Table 18 for experiments 1 and 2, Table 19 for experiments 3 through 5, Table 20 for experiments 6 through 12 and Table 21 for experiment 13.

Glucose--

Glucose levels were not significantly affected by CIO levels up to 18.3 μeq/l (0.65 mg/l) (ANOVA, $p > 0.10$, Table 18). Measured glucose levels at all dose levels including controls were low, approaching the detection limit of the analytical method, and were not comparable to levels reported by Lynch and Webb (1973c). Careful analysis of the method failed to explain the discrepancy. Values were consistently low regardless of method modifications or age of reagents. The discrepancy may have been the result of the high sensitivity of blood glucose to stress. Florkin (1960) cited blood glucose values of 175-182 mg % (cf. Lynch and Webb, 1973c) for freshly caught C. sapidus and values of 9.6-19.0 mg % (cf. Table 18) after one day of fasting. The low glucose values observed coupled with the reported high sensitivity of this blood constituent to any general stress indicated to us that blood glucose was not a suitable parameter to use in our laboratory study. Therefore blood glucose was not measured in subsequent experiments.

TNPS--

TNPS concentration was unaffected in the first experiment up to 18.3 μeq/l (0.65 mg/l) (ANOVA, $p > 0.10$; Table 18). In the second test, TNPS was unaffected at low doses but increased over 2000% at 27.4 μeq/l (0.97 mg/l), a dose very close to the 96-hr LC50 (ANOVA, $p < 0.005$, Table 18). In the fifth test, statistical analysis of the data was unproductive because of the small sample sizes and large variances encountered. The overlap between the standard errors precluded detection of significant differences. In spite of this, TNPS (Table 19) showed a slight increase in both controls and experimentals after two week exposure to 12.4 μeq/l (0.44 mg/l), the experimentals tending to have higher concentrations throughout the test.

In experiment 6, TNPS varied with time (ANOVA, $p < 0.005$; Table 20) but not with dose (10.2 μeq/l = 0.36 mg/l). Similar results were found for experiment 7 (11.8 μeq/l = 0.42 mg/l) (ANOVA, $p < 0.01$). In both tests an increase in TNPS was seen between days one and two, and, as in test 5, the experimental crabs seemed to maintain slightly higher TNPS levels than the control crabs.

TABLE 16. SUMMARY OF TEST SYSTEMS USED, DOSES, SAMPLING INTERVALS AND SERUM ANALYSES DURING *Callinectes sapidus* SEROLOGICAL STUDIES

Experiment no.	System	Mean measured CIO ($mgCl_2$/liter) with crabs	Sampling interval	No. crabs/sample (design)	Analyses
1	18-liter (5-gallon) aquaria	0.00, 0.11, 0.51, 0.65	4 days	5	Glucose, TNPS, Protein, Chloride, Osmotic
2		0.00, 0.17, 0.30, 0.97	4 days	5	TNPS, Protein, Chloride, Osmotic
3	48 compartment	0.00, 0.75	1 week	8 baseline; 4 control; 4 experimental	Protein, Chloride, Osmotic
4		0.00, 0.71	1 week	8 baseline; 4 control; 4 experimental	Protein, Chloride, Osmotic
5		0.00, 0.44	1 week	8 baseline; 4 control; 4 experimental	TNPS, Protein, Chloride
6	37-liter (10-gallon) aquaria	0.00, 0.36	1 day	10 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic
7		0.00, 0.42	1 day	10 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic

(continued)

TABLE 16 (continued)

Experiment no.	System	Mean measured ClO ($mgCl_2$/liter) with crabs	Sampling interval	No. crabs/sample (design)	Analyses
8	37-liter (10-gallon) aquaria	0.00, 0.47	1 day	10 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic
9		0.00, 0.50	1 day	10 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic
10		0.00, 0.48	1 day	10 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic
11		0.00, 0.62	1 day	10 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic
12		0.00, 1.04	1 day	6 baseline; 10 control; 10 experimental	TNPS, Protein, Chloride, Osmotic
13	37-liter (10-gallon) 1000 ml/min	0.00, 0.82	3 days	10 baseline; 10 control; 10 experimental	TNPS, Chloride, Osmotic
	18-liter (5-gallon) aquaria 500 ml/min	0.00, 0.74	3 days	5 control 5 experimental	TNPS, Chloride, Osmotic
	18-liter (5-gallon) aquaria 250 ml/min	0.00, 0.58	3 days	5 control 5 experimental	TNPS, Chloride, Osmotic

TABLE 17. SUMMARY OF HYDROGRAPHIC DATA FOR *Callinectes sapidus* SEROLOGICAL STUDIES

Experiment No.	Applied dose		Mean measured CIO level		Measured CIO standard deviation		Temperature (°C)		Salinity (⁰/oo)		Mean D.O. (mg/1)	Mean pH
	µeq/1	mg/1	µeq/1	mg/1	µeq/1	mg/1	$\overline{x}$	SD	$\overline{x}$	SD		
1	ND ND ND	ND ND ND	3.1 14.4 18.3	0.11 0.51 0.65	0.56 0.85 3.95	0.02 0.03 0.14	21.3	0.80	17.0	1.3	ND	ND
2	ND ND ND	ND ND ND	4.7 8.4 27.4	0.17 0.30 0.97	0.85 0.56 0.77	0.03 0.02 0.24	11.8	0.76	17.0	ND	ND	ND
3	63.5	2.5	21.2 (25.1)	0.75 (0.89)	7.61 (11.84)	0.27 (0.42)	18.4	0.77	18.5	0.28	6.2	7.6
4	60.6	2.15	20.0 (23.1)	0.71 (0.82)	3.38 (23.1)	0.12 0.82	20.2	0.94	18.4	0.34	6.7	7.8
5	54.7	1.94	12.4 (13.3)	0.44 (0.47)	1.97 (3.67)	0.07 (0.13)	27.2	0.81	20.3	0.87	4.8	7.7
6	36.4	1.29	10.2 (7.6)	0.36 (0.27)	0.85 (1.13)	0.03 (0.04)	20.9	0.21	19.3	1.3	6.7	7.8
7	43.7	1.55	11.8 (9.0)	0.42 (0.32)	1.13 (0.56)	0.04 (0.02)	21.11	0.32	18.6	0.72	6.7	7.8
8	46.2	1.64	13.3 (9.6)	0.47 (0.34)	1.97 (1.69)	0.07 (0.06)	23.0	0.62	17.1	0.42	6.6	8.0
9	55.3	1.96	14.1 (10.7)	0.50 (0.38)	0.56 (0.85)	0.02 (0.03)	21.8	0.68	19.4	0.42	6.4	7.9

(continued)

TABLE 17 (continued)

Experiment No.	Applied dose		Mean measured CIO level		Measured CIO standard deviation		Temperature (°C)		Salinity (/oo)		Mean D.O. (mg/1	Mean pH	
	µeq/1	mg/1	µeq/1	mg/1	µeq/1	mg/1	$\overline{x}$	SD	$\overline{x}$	SD			
10	69.4	2.46	13.5 (12.4)	0.48 (0.44)	1.41 (2.26)	0.05 (0.08)	29.0	0.60	21.9	0.43	5.6	7.6	
11	93.6	3.32	17.5 (23.1)	0.62 (0.82)	2.26 (5.92)	0.08 (0.21)	27.6	0.81	22.8	0.21	6.0	7.7	
12	120.7	4.28	29.3 (39.8)	1.04 (1.41)	9.87 (3.95)	0.35 (0.14)	26.7	0.65	24.3	0.14	6.4	7.8	
13	102.9	3.65	23.1 (32.4)	0.82 (1.15)	5.92 (4.51)	0.21 (0.16)	25.0	0.62	24.0	0.09	6.6	7.8	37-1 tank
	102.9	3.65	20.9 (32.7)	0.74 (1.16)	4.23 (4.79)	0.15 (0.17)	25.0	0.62	24.0	0.09	6.6	7.8	18-1 high flow
	129.2	4.58	16.4 (30.5)	0.58 (1.08)	1.97 (2.54)	0.07 (0.09)	25.0	0.62	24.0	0.09	6.6	7.8	18-1 tank low flow

ND No data
( ) Without crabs

TABLE 18. BLOOD SERUM PARAMETERS FOR *Callinectes sapidus* FROM EXPERIMENTS 1 AND 2.

Experiment	Temp. °C	CIO (mg/1)	Glucose (mg/100 ml) $\bar{x}$	Glucose (mg/100 ml) SD	Protein (mg/ml) $\bar{x}$	Protein (mg/ml) SD	Chloride (meq/1) $\bar{x}$	Chloride (meq/1) SD	TNPS (moles/ml) $\bar{x}$	TNPS (moles/ml) SD	Osmotic Conc. (milliosmols) $\bar{x}$	Osmotic Conc. (milliosmols) SD
1	21.3	0.00	7.3	1.5	71.4	24.7	392.1	20.8	2.9	1.4	864.2	15.0
	21.3	0.11	8.8	2.5	85.1	15.4	404.1	8.5	2.6	1.3	883.0	17.9
	21.3	0.51	8.9	6.3	63.7	32.1	386.8	19.7	2.8	1.2	834.0	45.9
	21.3	0.65	13.8	14.7	53.9	32.0	334.0	18.4	2.9	2.2	767.0	88.6
2	11.8	0.00	ND	ND	92.3	23.2	387.4	13.6	0.4	0.3	775.0	19.9
	11.8	0.17	ND	ND	66.8	46.7	392.8	121.7	0.4	0.3	785.1	21.4
	11.8	0.30	ND	ND	59.4	43.0	376.4	23.3	0.2	0.1	797.2	7.0
	11.8	0.97	ND	ND	58.5	23.3	259.8	13.5	8.1	1.0	613.8	35.7

ND = No data

TABLE 19. BLOOD SERUM PARAMETERS FOR *Callinectes sapidus* FROM EXPERIMENTS 3-5.

Experiment	Temp. °C	CIO (mg/1)	Weeks	TNPS (µmoles/ml) $\bar{x}$	TNPS (µmoles/ml) SD	Protein (mg/ml) $\bar{x}$	Protein (mg/ml) SD	Chloride (meq/1) $\bar{x}$	Chloride (meq/1) SD	Osmotic Conc. (milliosmols) $\bar{x}$	Osmotic Conc. (milliosmols) SD
3	18.4	0.00	1	ND	ND	73.5	27.3	372.4	18.6	845.0	82.2
	18.4	0.75	1	ND	ND	75.3	24.3	301.6	52.6	740.3	88.7
4	20.2	0.00	1	ND	ND	33.2	10.0	416.3	66.1	748.3	98.1
	20.2	0.71	1	ND	ND	54.8	16.0	300.6	52.6	699.0	59.3
5	27.2	0.00	1	1.5	0.7	26.4	18.3	387.2	13.5	ND	ND
	27.2	0.00	2	3.0	0.7	6.6	3.7	558.5	34.2	ND	ND
	27.2	0.44	1	2.6	1.0	44.3	32.4	373.2	59.7	ND	ND
	27.2	0.44	2	2.3	0.9	21.7	17.1	311.7	26.8	ND	ND

ND = No data

TABLE 20. BLOOD SERUM PARAMETERS FOR *Callinectes sapidus* FROM EXPERIMENTS 6-12.

Experiment	Temp. °C	CIO (mg/l)	Days	TNPS (µmoles/ml) $\bar{x}$	SD	Protein (mg/ml) $\bar{x}$	SD	Chloride (meq/l) $\bar{x}$	SD	Osmotic Conc. (milliosmols) $\bar{x}$	SD
6	20.9	0.00	1	2.0	0.3	61.3	15.6	397.2	8.5	786.4	23.2
	20.9		2	2.7	0.7	68.0	16.0	382.2	10.4	799.2	25.7
	20.9		3	2.4	0.4	65.3	15.2	380.1	17.8	791.6	20.4
	20.9		4	2.3	0.6	65.0	21.6	365.6	15.8	775.3	16.6
	20.9	0.36	1	1.6	0.4	56.4	16.1	398.9	18.4	804.6	27.2
	20.9		2	2.9	0.9	67.1	22.7	388.7	5.5	831.5	28.9
	20.9		3	2.8	0.6	56.1	13.0	381.2	12.7	811.8	25.9
	20.9		4	2.6	0.6	63.0	24.7	371.7	11.9	788.1	11.3
7	21.1	0.00	1	2.6	0.7	67.4	19.9	370.0	12.8	739.3	18.8
	21.1		2	3.3	0.6	69.6	29.0	364.5	9.7	757.6	19.1
	21.1		3	3.2	0.9	63.6	16.0	364.8	10.9	746.4	13.7
	21.1		4	3.4	0.7	72.5	15.0	363.9	9.9	769.8	12.0
	21.1	0.42	1	2.8	0.5	67.5	15.3	382.2	11.4	762.2	22.6
	21.1		2	3.5	1.3	72.4	21.0	373.8	14.5	763.0	22.2
	21.1		3	3.2	0.9	61.8	20.3	369.7	18.1	745.8	27.7
	21.1		4	4.0	1.3	78.2	22.7	364.7	10.9	763.1	19.3
8	23.0	0.00	1	3.0	0.4	52.0	20.6	361.1	24.1	774.0	21.2
	23.0		2	3.4	1.0	56.7	27.4	384.4	21.2	776.7	24.5
	23.0		3	2.6	1.4	52.5	10.4	373.5	16.4	737.5	25.3
	23.0		4	2.9	1.0	47.3	9.5	393.2	33.7	733.6	52.2
	23.0	0.47	1	2.5	0.8	42.2	16.1	388.0	48.4	762.5	35.5
	23.0		2	3.2	1.5	56.2	23.8	371.6	28.0	775.0	41.0
	23.0		3	2.5	1.1	56.9	21.8	383.8	15.0	755.2	41.2
	23.0		4	2.4	1.2	37.4	16.7	390.6	48.2	748.0	40.7

Table 20. (continued)

Experiment	Temp. °C	CIO (mg/l)	Days	TNPS (µmoles/ml)		Protein (mg/ml)		Chloride (meq/l)		Osmotic Conc. (milliosmols)	
				$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
9	21.8	0.00	1	2.2	1.4	50.0	23.3	401.9	34.0	769.2	18.4
	21.8		2	2.9	1.0	45.0	20.8	382.0	14.7	761.1	10.9
	21.8		3	2.4	0.9	54.8	24.8	379.0	10.5	774.4	26.1
	21.8		4	2.2	1.1	45.4	27.0	377.9	5.4	775.0	28.8
	21.8	0.50	1	1.9	0.5	44.8	17.7	420.3	17.9	799.2	43.8
	21.8		2	3.2	1.2	57.6	20.8	386.1	10.4	799.3	35.7
	21.8		3	2.4	1.0	46.6	9.2	382.3	25.8	804.1	26.9
	21.8		4	2.5	1.3	53.4	20.0	381.6	16.2	789.2	30.6
10	29.0	0.00	1	3.1	0.5	49.2	15.5	418.7	47.5	776.6	32.6
	29.0		2	2.7	1.0	50.7	17.5	371.8	11.5	774.5	23.1
	29.0		3	3.3	1.0	61.2	19.0	381.5	19.9	765.9	37.9
	29.0		4	1.8	0.9	50.4	22.8	369.5	12.5	775.5	16.2
	29.0	0.48	1	2.9	0.7	46.8	17.3	425.8	42.2	798.5	34.9
	29.0		2	2.8	0.5	51.1	20.3	395.7	13.3	808.5	45.6
	29.0		3	2.7	0.5	55.4	15.9	390.3	10.4	787.9	29.0
	29.0		4	2.9	1.0	47.4	19.4	377.0	8.1	817.2	51.6
11	27.6	0.00	1	3.0	0.8	38.4	21.5	389.0	7.4	782.2	33.7
	27.6		2	2.7	0.7	35.3	11.5	380.1	12.0	804.5	60.1
	27.6		3	4.3	1.4	45.7	25.3	371.1	8.7	798.7	23.3
	27.6		4	3.2	0.8	34.5	9.3	379.5	15.5	786.1	20.2
	27.6	0.62	1	2.5	0.8	32.1	15.7	398.5	6.3	803.4	22.5
	27.6		2	3.7	2.1	33.6	13.9	388.5	7.3	818.8	33.6
	27.6		3	2.8	0.8	34.3	12.7	377.4	17.4	814.0	32.3
	27.6		4	3.4	2.7	39.0	25.7	371.0	14.4	794.6	27.1

Table 20. (concluded)

Experiment	Temp. °C	CIO (mg/1)	Days	TNPS (μmoles/ml) $\bar{x}$	TNPS (μmoles/ml) SD	Protein (mg/ml) $\bar{x}$	Protein (mg/ml) SD	Chloride (meq/1) $\bar{x}$	Chloride (meq/1) SD	Osmotic Conc. (milliosmols) $\bar{x}$	Osmotic Conc. (milliosmols) SD
12	26.7	0.00	1	2.9	1.3	22.0	7.6	396.4	10.8	775.6	17.9
	26.7		2	2.6	1.2	32.9	10.7	397.6	10.9	792.1	25.4
	26.7	1.04	1	3.2	1.0	26.0	12.5	385.8	15.3	796.9	32.0
	26.7		2	3.1	0.6	34.4	14.1	383.1	13.8	780.1	14.4

TABLE 21. BLOOD SERUM PARAMETERS FOR *Callinectes sapidus* FROM EXPERIMENT 13.

Tank; flow	Temp. °C	CIO (mg/1)	TNPS (µmoles/ml)		Chloride (meq/1)		Osmotic Conc. (milliosmols)	
			$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
37 liter; high flow	25	0.00	2.9	0.5	391.6	9.3	783.4	16.2
18 liter; high flow	25	0.00	2.9	0.7	402.6	9.0	786.4	25.0
18 liter; low flow	25	0.00	2.7	1.3	403.2	7.5	780.8	17.4
37 liter; high flow	25	0.82	3.6	0.7	371.0	14.9	738.9	3.2
18 liter; high flow	25	0.74	3.3	0.5	360.9	6.8	741.8	21.6
18 liter; low flow	25	0.58	1.9	0.7	398.6	8.3	797.3	10.4

TNPS was unaffected by either dose or time in experiments 8 (13.3 μeq/l = 0.47 mg/l) (ANOVA, $p > 0.10$) and 9 (14.1 μeq/l = 0.50 mg/l) (ANOVA $p > 0.10$). However, trends with time seemed similar to those noted for experiments 6 and 7, and the experimental crabs in experiment 8 seemed to maintain slightly lower TNPS levels than the control crabs.

TNPS was affected by time but not dose (13.5 μeq/l = 0.48 mg/l) in experiment 10 (ANOVA, $p < 0.025$). The overall trend was not readily obvious and may reflect fluctuations in control values, even though there was no significant interaction between dose and time ($p > 0.05$).

TNPS was not influenced by dose or time in experiments 11 (17.4 μeq/l = 0.62 mg/l) (ANOVA, $p > 0.10$) and 12 (29.3 μeq/l = 1.04 mg/l) (ANOVA, $p > 0.10$). However, as in some of the previous tests, experimental crabs in the twelfth test seemed to maintain slightly higher TNPS levels than control crabs.

TNPS levels did not vary with the tank size-flow-CIO combinations of experiment 13 (ANOVA, $p > 0.10$; Table 21). The ANOVA was supported by Tukey's modified ω' (Table 22).

Protein--

Protein in the first two tests appeared to decrease with increasing CIO level (Table 18), but the decrease was not significant (ANOVA, $p > 0.10$). In addition, there was no effect at the higher CIO doses of tests 3 (21.2 μeq/l = 0.75 mg/l) and 4 (20.0 μeq/l = 0.71 mg/l) ($t = -0.10, -2.26$ respectively; $p > 0.05$). In the fifth test serum protein showed a tendency to be elevated by exposure to CIO levels (12.4 μeq/l = 0.44 mg/l) (Table 19). However, as with TNPS, the small sample sizes and high variability in the data precluded meaningful statistical analysis. The apparent decreases in protein over the two-week period in this test and over the four-day period in tests 1 and 2 may be attributable to inadequate feeding. Serum protein in experiments 6 through 12 varied with neither dose nor time (ANOVA, $p > 0.05$; Table 20). Therefore, protein was not determined for experiment 13.

Chloride--

At low doses in the first two experiments serum chloride levels showed a slight increase in concentration, but as the dose was increased above 8.5-14.1 μeq/l (0.3-0.5 mg/l), the serum chloride level dropped markedly (Table 18). The effect on serum chloride was highly significant in spite of high variance in the data. Similar trends were apparent in the third and fourth experiments ($t = 2.54, 2.74$, respectively; $p < 0.05$) and, although not statistically verifiable, in experiment 5 (Table 19). In experiment 3 chloride was

TABLE 22. TUKEY's MODIFIED ω' TEST FOR TANK SIZE-FLOW-CIO COMBINATION EFFECTS ON SERUM TNPS IN *Callinectes sapidus* FROM EXPERIMENT 13. [$\omega' = q\alpha(P,N_2)S$; STEEL AND LORRIE, 1960] BARS UNDERLINE EQUAL MEANS (α=0.05)

tank size (liters)	18	18	37	18	18	37
flow rate (ml/min)	250	250	1000	500	500	1000
CIO level ($mgCl_2/l$)	0.58	0.00	0.00	0.00	0.74	0.82
TNPS $\bar{x}$ (μmoles/ml)	1.92	2.70	2.89	2.91	3.34	3.56
Equal means	———	———	———	———	———	———

TABLE 23. TUKEY's MODIFIED ω' TEST FOR TANK SIZE-FLOW-CIO COMBINATION EFFECTS ON SERUM CHLORIDE IN *Callinectes sapidus* FROM EXPERIMENT 13. [$\omega' = q\alpha(P,N_2)S$; STEEL AND TORRIE, 1960] BARS UNDERLINE EQUAL MEANS (α=0.05)

tank size (liters)	18	37	37	18	18	18
flow rate (ml/min)	500	1000	1000	250	500	250
CIO level ($mgCl_2/l$)	0.74	0.82	0.00	0.58	0.00	0.00
chloride (meq/liter)	360.9	371.0	391.6	398.6	402.6	403.2
Equal means			———	———	———	———
		———	———			
	———	———				

TABLE 24. TUKEY's MODIFIED ω' TEST FOR TANK SIZE-FLOW-CIO COMBINATION EFFECTS ON SERUM OSMOTIC CONCENTRATION IN *Callinectes sapidus* FROM EXPERIMENT 13. [$\omega' = q\alpha(P,N_2)S$; STEEL AND TORRIE, 1960] BARS UNDERLINE EQUAL MEANS (α= 0.05)

tank size (liters)	37	18	18	37	18	18
flow rate (ml/min)	1000	500	250	1000	500	250
CIO level ($mgCl_2/l$)	0.82	0.74	0.00	0.00	0.00	0.58
osmotic concentration (milliosmols)	738.9	741.8	780.8	783.4	786.4	797.3
Equal means	———	———	———	———	———	———

reduced from the baseline value of 390.0 meq/l to 301.7 meq/l in the experimentals compared to a control reduction to 372.4 meq/l at 21.2 μeq/l (0.75 mg/l) CIO. In experiment 4 the chloride in the experimentals was reduced from 393.7 meq/l to 300.6 meq/l, while the controls showed an increase to 416.3 meq/l at 20.0 μeq/l (0.71 mg/l) CIO. Similarly, while chloride concentrations in the hyperregulating control crabs of experiment 5 conformed to changes in chloride ion in the medium and increased after two weeks, the levels in the experimental crabs dropped markedly at 12.4 μeq/l (0.44 mg/l).

Though rarely significant, serum chloride levels in most subsequent tests showed a tendency to be elevated in crabs exposed to CIO. Serum chloride levels in the sixth test were affected only by time (ANOVA, $p < 0.005$; Table 20), decreasing over the four-day period. Those in the seventh and eighth experiments were unaffected by either dose or time (ANOVA, $p > 0.10$). In experiment 9, chloride levels were affected by time (ANOVA, $p < 0.005$), decreasing from the first to the second day before leveling out in both experimentals and controls. Serum chloride in the tenth test varied with both dose and time (ANOVA, $p < 0.005$). Chloride levels decreased from the first to the fourth day and were consistently higher in the dosed crabs. Serum chloride in experiment 11 changed only with time (ANOVA, $p < 0.05$), showing a gradual decrease from day 1 to day 4. In experiment 12 serum chloride was affected only by dose (ANOVA, $p < 0.01$), experimental crabs having lower levels than the controls.

Serum chloride levels varied with tank size-flow-CIO combinations in experiment 13 (ANOVA, $p < 0.005$; Table 21). Essentially, chloride levels in crabs from the 37-liter-1000 ml/min-23.1 μeq/l (0.82 mg/l) and 18-liter-500 ml/min-20.9 μeq/l (0.74 mg/l) combinations were equivalent to each other and lower than those in all other combinations (Table 23).

Osmotic--

Serum osmotic concentrations, not surprisingly, followed trends similar to serum chloride. In the first two tests, osmotic concentrations showed a slight increase at low chlorine residual doses, and then dropped radically at doses above 8.5-14.1 μeq/l (0.3-0.5 mg/l) (Table 18). The effect of the CIO levels on serum osmotic concentration was highly significant. It was surprising, therefore, that at the higher doses of tests 3 (21.2 μeq/l = 0.75 mg/l) and 4 (20.0 μeq/l = 0.71 mg/l) no effect was evident ($t = 1.73$, 0.86, respectively; $p > 0.05$). Samples for determining serum osmotic concentrations for the fifth test were lost because of a malfunctioning osmometer.

Like serum chloride, serum osmotic concentrations in subsequent tests showed usually nonsignificant but consistent increases in crabs exposed to CIO. Serum osmotic concentrations in the sixth experiment were dependent on both dose and time (10.2 μeq/l = 0.36 mg/l) (Table 20). The experimental crabs had consistently higher values than the controls as all values increased from the first to the second day and then steadily declined. In the seventh test, osmotic concentration was dependent only on time. Apparent minor fluctuations with time were found significant, probably because of the unusually low variability of the data. With the exception of experiment 9 osmotic concentration varied with neither dose nor time from experiments 8 through 12 (ANOVA, $p > 0.05$). In the ninth test osmotic concentration was affected by dose, showing higher levels in the experimental crabs than in the controls on all days (ANOVA, $p < 0.005$).

The effect of the tank size-flow-CIO combinations of experiment 13 on osmotic concentration (ANOVA, $p < 0.005$; Table 21) was similar to that observed for serum chloride levels. Osmotic concentrations in crabs from the 37-liter-1000 ml/min-23.1 μeq/l (0.82 mg/liter) and 18-liter-500 ml/min-20.9 μeq/l (0.74 mg/liter) combinations were equivalent to each other and lower than those in all other combinations (Table 24).

Behavioral and Other Responses

Feeding--

Crabs were fed throughout experiments 5 through 12 with either an experimental Purina Marine Chow or squid squares. Disintegration of the marine chow in the fifth test after wetting and manipulation or trampling by the crabs made it difficult to be certain whether there were feeding differences between experimentals and controls. It seemed, however, that the experimentals were feeding less. This was confirmed during tests 6 through 12 in which experimental crabs obviously ate less squid than the controls. Whether this feeding response was the result of ill health in the experimental crabs or an inability on their part to sense the food in chlorinated water is unclear. A clue may have been offered in experiment 9 in which experimental crabs began feeding immediately (1-2 hr) after chlorine input to the dosing system was accidently shut off.

Spawning--

In the fifth experiment of this section, several of the crabs spawned in the 48-compartment system. Virtually all of the observed spawning occurred in the controls. Retention of eggs on the pleopods was poor probably because of the confinement.

Activity--

Effects of CIO levels on crab motility were unobservable because the dosing system was too confining. However, it was noticed that dosed crabs tended to keep their antennules withdrawn, making only a few tentative movements into the water. This reaction seemed more pronounced at higher doses and contrasted with the control crabs whose antennules seemed to be always extended.

Chlorine Demand

During tests using the 48-compartment system, it was noticed that introduction of crabs to the system seemed to alter the measured CIO concentrations. Specifically, at high CIO concentrations introduction of crabs lowered the measured level. Such a demand was present in the fifth test where the CIO concentration was reduced from 13.3 μeq/l (0.47 mg/l) to 12.4 μeq/l (0.44 mg/l) when the crabs were present ($t = 2.46$, < 0.02). It also occurred in tests 3 and 4 but was significant only in test 4 where the CIO level was reduced from 23.1 μeq/l (0.82 mg/l) to 20.0 μeq/l (0.71 mg/l) ($t = 3.99$, < 0.001). This phenomenon occurred also in the tests for lethal limits but was attributed to varying demand in the incoming water. In the 48-compartment system, however, the crabs were implicated because the pattern was not immediately visible except at the higher doses. In one test, measured CIO levels in tanks with and without crabs were compared statistically for a series of doses ranging from approximately 0.85 to 14.1 μeq/l (0.03 to 0.5 mg/l) (Table 25). Analysis revealed a demand only at the highest level where crabs reduced the measured CIO concentrations from 13.8 to 11.8 μeq/l (0.49 to 0.42 mg/l). This demand was thought to be the result of the excretion of ammonia or organics by the crabs. It was thought unusual, therefore, and perhaps anomalous that at the next lower dose (5.6-7.6 μeq/l = 0.20-0.27 mg/l) introduction of crabs raised the measured CIO level.

This latter phenomenon turned out not to be unusual at all. A retrospective look at Table 25 shows that, although differences were not significant at individual doses, compartments with crabs consistently showed higher measured CIO levels at the lower doses than those without. Also, in tests 6 through 10 introduction of crabs to the system caused a marked increase in the measured CIO level that persisted throughout the tests (Table 17). Differences were significant ($t = 11.2, 11.5, 8.5, 14.6$ and 2.5, respectively; $p < 0.02$). The effect was reversed in experiment 11 ($t = 4.7$; $p < 0.001$) and apparently in experiments 12 and 13, although simultaneous comparisons among tanks with and without crabs were not possible in these latter tests.

It seems as if crabs increase the measured CIO level at low doses but decrease it at high doses approaching and exceeding the level of no effect (18.3 μeq/l = 0.65 mg/l) derived from the 96-hr LC50 tests. Determination of the mechanisms responsible for this effect is beyond the scope of this study, but we hypothesize that ammonia produced by the crabs ties up the CIO as monochloramine,

TABLE 25. COMPARISON OF CIO LEVELS IN *Callinectes* DOSING SYSTEM WITH AND WITHOUT CRABS

Compartment	Tank 1	2	3	4	5	6
A	0.03	0.07*	0.12	0.23*	0.50	0.00*
B	0.03*	0.07	0.11*	0.30*	0.50	0.00
C	0.04	0.08*	0.11	0.19	0.41*	0.00*
D	0.04*	0.08	0.11	0.20	0.48	0.00
E	0.04*	0.05	0.11*	0.28*	0.43*	0.00
F	0.04	0.07	0.12*	0.19	0.45*	0.00
G	0.05*	0.07*	0.12*	0.25*	0.37*	0.00
H	0.03	0.07*	0.10	0.20	0.46	0.00*
without crab						
$\bar{X}$	0.033	0.068	0.110	0.195	0.485	0.000
SD	0.005	0.013	0.008	0.006	0.019	0.000
CV	15	18	7	3	4	0
with crab						
$\bar{X}$	0.04	0.073	0.115	0.265	0.415	0.000
SD	0.008	0.005	0.006	0.031	0.034	0.000
CV	20	7	5	12	8	0
t	1.5666 ns	0.7385 ns	0.9999 ns	4.43**	3.575**	
Applied Dose	30.8 mg/1	61.6 mg/1	92.4 mg/1	123.2 mg/1	154.0 mg/1	0

* with crab

which in turn contributes to higher measured CIO levels. Positive demand at higher doses could represent bounding of chlorine residuals to organic compounds released by stressed crabs.

Ammonia Nitrogen

Tank NH_3-N levels were dependent on the tank size-flow-CIO combinations of experiment 13 (ANOVA, $p < 0.005$). As would be expected the highest levels occurred in the tanks with the lowest flow per crab (18-liter-250 ml/min) (Table 26). The remaining tanks, all with equivalent flows per crab, had roughly comparable NH_3-N levels within each of the control and experimental groups. Measured NH_3-N levels were greatly reduced in tanks containing CIO. The mean diluent NH_3-N level ($\bar{x} = 0.10$ mg/l; SD = 0.04) was less than half the lowest level of the control group ($\bar{x} = 0.25$ mg/l; SD = 0.06).

TABLE 26. TUKEY's ω-TEST FOR TANK SIZE-FLOW-CIO COMBINATION EFFECTS ON AMMONIA-NITROGEN IN TANKS FROM EXPERIMENT 13 BARS UNDERLINE EQUAL MEANS (α=0.05)

tank size (liters)	37	18	18	37	18	18
flow rate (ml/min)	1000	500	250	1000	500	250
CIO level ($mgCl_2/l$)	0.82	0.74	0.58	0.00	0.00	0.00
NH_4-N (mg/l)	0.01	0.03	0.12	0.25	0.32	0.49
Equal means	———	———	———	———	———	

SECTION 7

RESPIRATORY EFFECTS

METHODS

Experimental Analysis

Mature female blue crabs for study of the short-term effects of CIO on oxygen consumption were obtained from the Chesapeake Bay potting fishery. Mature females for all other studies came from the Chesapeake Bay dredge fishery. Again, the nature of the fisheries makes it difficult to determine the precise temperature and salinity regimes from which the crabs were taken. Potted crabs probably came from salinities between 20 and 25 °/oo and temperatures of 15 to 20°C. Dredged crabs came from higher salinities (20-30 °/oo) and lower temperatures (< 5°C). No attempt has been made to compare physiological measurements between the two populations, but crabs from only one group have been used for a given series of tests.

Prior to their use all crabs were held for 4-7 days in the recirculating sea water system used in the serological study. Crabs were held at room temperature (18-20°C for those used in the short-term test; 14-17°C for those used in subsequent tests) and York River salinities (19.5 to 21.8 °/oo and 15.1 to 19.4 °/oo, respectively). All crabs were fed squid daily, and their claws were bound to prevent cannibalism.

Exposure Systems

The basic dilutor system was similar to that described in detail in Section 4.

Short-term effects of CIO on blue crab oxygen consumption were determined directly in a continuous-flow respirometer. Each of two cylindrical, three-liter chambers for individual crabs was fitted at the top with a large rubber stopper containing a tapered opening for a self-stirring B.O.D. oxygen probe, an incurrent tube and an excurrent tube. Rate of flow through each plexiglas chamber could be adjusted by raising or lowering the excurrent tube. Incurrent water was supplied from a header box which received water from a mixing box. Appropriate CIO levels were obtained by pumping stocks of varied chlorine concentrations into the mixing box and by adjusting the diluent flow (2040 ml/min for high doses, 3000 ml/min for low doses).

Crabs in all remaining tests were exposed to ClO in 37-liter tanks. Crabs in six aquaria served as controls while crabs in another six aquaria were dosed with ClO.

Experimental Protocols

Short-term effects of ClO on the oxygen consumption of whole crabs were determined by monitoring oxygen levels (mg/l) in the 3-liter chambers while individual crabs were being exposed to ClO. Ten crabs each were exposed to a high and a low dose. After crabs were introduced to the chambers, excurrent flow was monitored and adjusted to ~100 ml/min. Oxygen levels in the chambers were monitored continuously, using YSI 5420A oxygen probes and YSI 54 oxygen meters attached to a YSI 81A dual channel recorder. The crabs were exposed to unchlorinated water for 2 hr from the time the oxygen concentration in both chambers became relatively constant. The water was then chlorinated for 2 hr. At the end of this period, chlorination was stopped; ClO levels in the chambers were allowed to decrease to undetectable levels; and the crabs were exposed to unchlorinated water for a final 2 hr. Throughout each test, ambient oxygen levels in the header tanks were monitored at 15-min intervals. Excurrent flow rates were determined every 30 min. Temperature was measured at the beginning and end of each test along with salinity and at the beginning and end of each chlorination period. ClO was measured at 7.5-min intervals during and after chlorination until levels became undetectable. After each test, the crabs were blotted dry and weighed.

Remaining longer-term tests used twelve 37-liter tanks. The design called for sixty crabs (ten per tank) to be subjected to a single ClO level with sixty others serving as controls. Total exposure time was four days followed by a recovery period of four days. At two, four, six, and eight days, preselected random samples of nine dosed and nine control crabs were extracted for determination of oxygen uptake. In one test employing only one experimental and one control tank, preselected samples of five crabs were taken after one day. ClO levels, temperature, salinity, dissolved oxygen, and mortality were measured three times daily; pH and flow rates were monitored once daily.

Respirometry

Respirometry for the continuous-flow system has already been described.

The respirometer used to determine whole animal oxygen consumption with the longer-term tests was a static system. Ten cubical plexiglas animal chambers (volume $\bar{x}$ = 2930 ml; SD = 8.8) were submerged in a water bath. To maintain acclimation conditions the water bath was continuously supplied with unchlorinated diluent water from the dosing system. Each animal chamber was fitted at the top with a rubber stopper containing an opening fashioned from a B.O.D. bottle neck.

At the beginning of each test, a crab from the preselected random sample of nine was put into each chamber and was allowed to adjust to the apparatus for 30 min. A tenth chamber was used as a blank. The chambers were isolated with dark plastic partitions. During the adjustment period, the chambers were aerated and open to the water bath. After the adjustment period, a rubber stopper was inserted into the first chamber along with a YSI 5720 self-stirring B.O.D. oxygen probe attached to a YSI 54 oxygen meter. A dissolved oxygen (mg/l) reading was taken following equilibration by the probe. The probe was then moved to the second chamber following insertion of its stopper. The first chamber was plugged with a B.O.D. bottle stopper. The procedure was repeated until rubber stoppers were in all the chambers. Subsequent readings from each chamber required only the removal of the B.O.D. bottle stopper and insertion of the probe. Readings were made in sequence through five cycles. Cumulative time from the beginning of each test was recorded at each reading. After each test, the crabs were blotted dry and weighed.

Following each determination of whole crab oxygen consumption, the same crabs were used to determine gill respiration on a Gilson differential respirometer. The second gill from the posterior (pleurobranch) was removed from the right side of each crab. Each gill, rinsed in 1 μm-filtered York River water, was placed in a 15 ml flask containing 5 ml unchlorinated 1 μm-filtered York River water in the bottom and ∿1 ml of 10% KOH in the side arm. After attachment to the respirometer, flasks were allowed 30 min to equilibrate at 16°C. A shaker frequency of 80 strokes per minute was used. Following equilibration, standard Gilson techniques were used and cumulative readings of μl of oxygen consumed were made every 10 min for 90 min. At the end of each test, the gills were blotted and dried to constant weight.

Histology

When gills were removed for the determination of their oxygen consumption, the opposing gill from the left side of each crab was preserved in Dietrich's fixative for sectioning and histological examination. Simultaneously, miscellaneous gills were examined fresh under 430 X magnification.

Data Analysis

Ambient oxygen levels were plotted on individual recordings (mg/l O_2 vs. time) from the continuous-flow respirometers. Each recording was then divided into five sections including the initial unchlorinated period, periods of rising CIO, steady CIO and declining CIO and a final unchlorinated period of recovery. Areas between ambient and chamber oxygen levels were then determined for each section on a Numonics model 237 graphics calculator (electronic planimeter). Oxygen consumption rate was computed for each section from the following formula:

$$O_2 \text{ consumption } [\mu l/g(\text{wet})/hr] = \frac{41940\ A\ C\ F}{WT}$$

A (cm^2): section area from planimeter
C [(mg) (min)/1000 ml]: recording units of section area (calibration factor)
F (ml/min): flow rate through chamber
W (g): crab wet weight
T (min): total section minutes
41940 = conversion factor (0.699 ml/mg x 1000 µl/ml x 60 min/hr)

Within each dose level all possible comparisons were made among the initial unchlorinated (control) period, the level CIO period and the unchlorinated recovery period using Student's t-test for paired observations.

Regressions of chamber oxygen levels (mg/l) versus time (min) were used to determine oxygen consumption rates for individual crabs in the static respirometers according to the following formula:

$$O_2 \text{ consumption } [\mu l/g(\text{wet})/hr] = \frac{41940\ [S(V_t - V_c) - B]}{W}$$

S (mg/1000 ml/min): slope of regression: x=min; y=mg/1000 ml
V_t (ml): chamber volume with stopper inserted
V_c (ml): calculated crab volume (Laird, unpublished)
B (mg/min): rate of change of oxygen content of blank chamber
W (g): crab wet weight

Differences between experimental and control crabs were determined for each sampling period by using Student's t-test for independent observations.

Regression of oxygen consumed (µl) versus time (hr) were used to determine rates of oxygen consumption for individual gills. Slopes (µl/hr) were converted to µl dry gas at 760 mm H_g and inserted into the following formula:

$$O_2 \text{ consumption } [\mu l/g(dry)/hr] = \frac{S_g - S_b}{W}$$

S_g (μl/hr): slope of regression for gill: x=hr; y=μl
S_b (μl/hr): slope of regression for blanks: x=hr; y=μl
W (g): gill dry weight

Differences in oxygen consumed by gills of experimental and control crabs were determined for each sampling period by using Student's t-test for independent observations.

RESULTS

Short-Term Effects

A summary of test systems can be found in Table 27. CIO levels and other hydrographic data appear in Table 28.

At the low dose (17.8 μeq/l = 0.63 mg/l) in the first experiment oxygen consumption did not change significantly from the initial unchlorinated period (135.5 μl/g/hr; SD = 29.3) to the CIO plateau period (119.0 μl/g/hr; SD = 30.8) ($t = 2.53$; $p > 0.02$) but showed a decrease from both of these to the recovery period (97.3 μl/g/hr; SD = 17.0) ($t = 6.27, 3.62$, respectively; $p < 0.01$).

At the high dose (22.3 μeq/l = 0.79 mg/l) in the second experiment oxygen consumption decreased from the initial period (134.7 μl/g/hr; SD = 20.8) to the plateau period (114.9 μl/g/hr; SD = 14.7) ($t = 3.11$; $p < 0.02$) and to the recovery period (114.4 μl/g/hr; SD = 17.6) ($t = 4.12$; $p < 0.01$). There was no difference, however, between values from the plateau and recovery periods ($t = 0.09$; $p > 0.5$).

Several tests were performed without chlorination to determine whether time alone was responsible for observed changes in oxygen consumption. Oxygen consumption did not vary significantly among time periods equivalent to those compared in the chlorinated tests ($p > 0.05$).

Longer-Term Effects

In the third (one day) test, using the static respirometers, crabs were exposed to 8.7 μeq/l (0.31 mg/l). Oxygen consumption of exposed crabs (44.7 μl/g/hr; SD = 10.4) was not significantly different from that of the controls (59.8 μl/g/hr; SD = 14.2) ($t = 1.92$; $p > 0.05$).

In the fourth and fifth experiments crabs were exposed to 7.6 μeq/l (0.27 mg/l) and 27.9 μeq/l (0.99 mg/l), respectively. In the fourth experiment oxygen consumption did not differ between experimental and control crabs either during the exposure period ($t = 0.29, 1.09$ for the two sampling days, respectively; $p > 0.2$) or during

TABLE 27. SUMMARY OF TEST SYSTEMS USED, DOSES, SAMPLING INTERVALS AND ANALYSES DURING *Callinectes sapidus* RESPIRATION STUDIES

Experiment No.	System (Respirometer)	Mean Measured CIO ($MgCl_2$/liter)	Sampling Interval	No. crabs/sample (Design)	Analysis
1	flow-through	0.63	continuous	10	whole crab oxygen consumption
2		0.79	continuous	11	whole crab oxygen consumption
3	static	0.31	1 day	5 experimental 5 control	whole crab oxygen consumption
4	static (whole crab) Gilson (Gill)	0.27	2 days	9 experimental 9 control	whole crab and excised gill oxygen consumption
5		0.99	2 days	9 experimental 9 control	whole crab and excised gill oxygen consumption

TABLE 28. SUMMARY OF HYDROGRAPHIC DATA FOR *Callinectes sapidus* RESPIRATION STUDIES

Experiment No.	Mean applied		Mean measured CIO level		Measured CIO standard deviation		Temperature (°C)		Salinity (°/oo)		Mean D.O. (mg/1)	Mean pH
	μeq/1	mg/1	μeq/1	mg/1	μeq/1	mg/1	$\bar{x}$	SD	$\bar{x}$	SD		
1	88.6	3.14	17.8	0.63	1.2	0.04	21.5	0.78	20.5	0.41	7.8 (Header)	ND
2	124.1	4.40	22.3	0.79	1.7	0.06	21.2	0.95	19.5	0.25	7.9 (Header)	ND
3	33.0	1.17	8.7	0.31	0.6	0.02	17.3	0.71	18.4	0.29	8.5	7.8
4	31.3	1.11	7.6	0.27	1.2	0.04	15.8	0.58	13.8	0.88	8.8	7.7
5	79.5	2.82	27.9	0.99	4.2	0.15	14.5	0.69	14.6	0.36	8.8	7.8

ND - No data.

the recovery period ($t = 0.05; -0.65; p > 0.5$) (Fig. 14). Gill respiration was also unaffected ($t = 0.13, 1.25, 0.22, -0.83$, respectively, for the four sampling days; $p > 0.2$) (Fig. 15). In the fifth test, no sample was taken on the fourth recovery day because of high mortalities among the experimental crabs. Experimental crabs from the first sample taken during chlorination showed higher whole crab oxygen consumption (52.0 µl/g/hr; SD = 6.3) than did the controls (35.5 µl/g/hr; SD = 9.3) ($t = 4.39; p < 0.001$) (Fig. 16). There was, however, no effect on the last day of chlorination ($t = 0.38; p > 0.5$) or on the second day of recovery ($t = -1.26; p > 0.2$). Gill respiration was unaffected throughout the test ($t = -1.63, -0.63, 1.04$, respectively, for the three sampling days; $p > 0.1$) (Fig. 17).

Histopathology

Fresh Material--

Gills from crabs exposed to 27.9 µeq/l (0.99 mg/l) had a blanched appearance after two days. Microscopic examination of individual platelets showed an optically denser, possibly thickened area at the center. Extent of this area varied somewhat among gills. In addition, the normally reticular pattern of cells in the outer portions of the platelets seemed disrupted. These effects persisted into the recovery period.

Fixed Material--

Gill tissues were fixed in Dietrich's fixative, stained with Mayer's hematoxylin, Astra blue and Eosin Y, and sectioned. Examination of gill tissues with a light microscope revealed no pathological changes.

Activity

The continuous-flow tests allowed a crude estimate to be made of the amount of CIO required for crabs to withdraw their antennules. Permanent withdrawal was caused by 6.2 µeq/l (0.22 mg/l) at 21.5 ± 1.5°C.

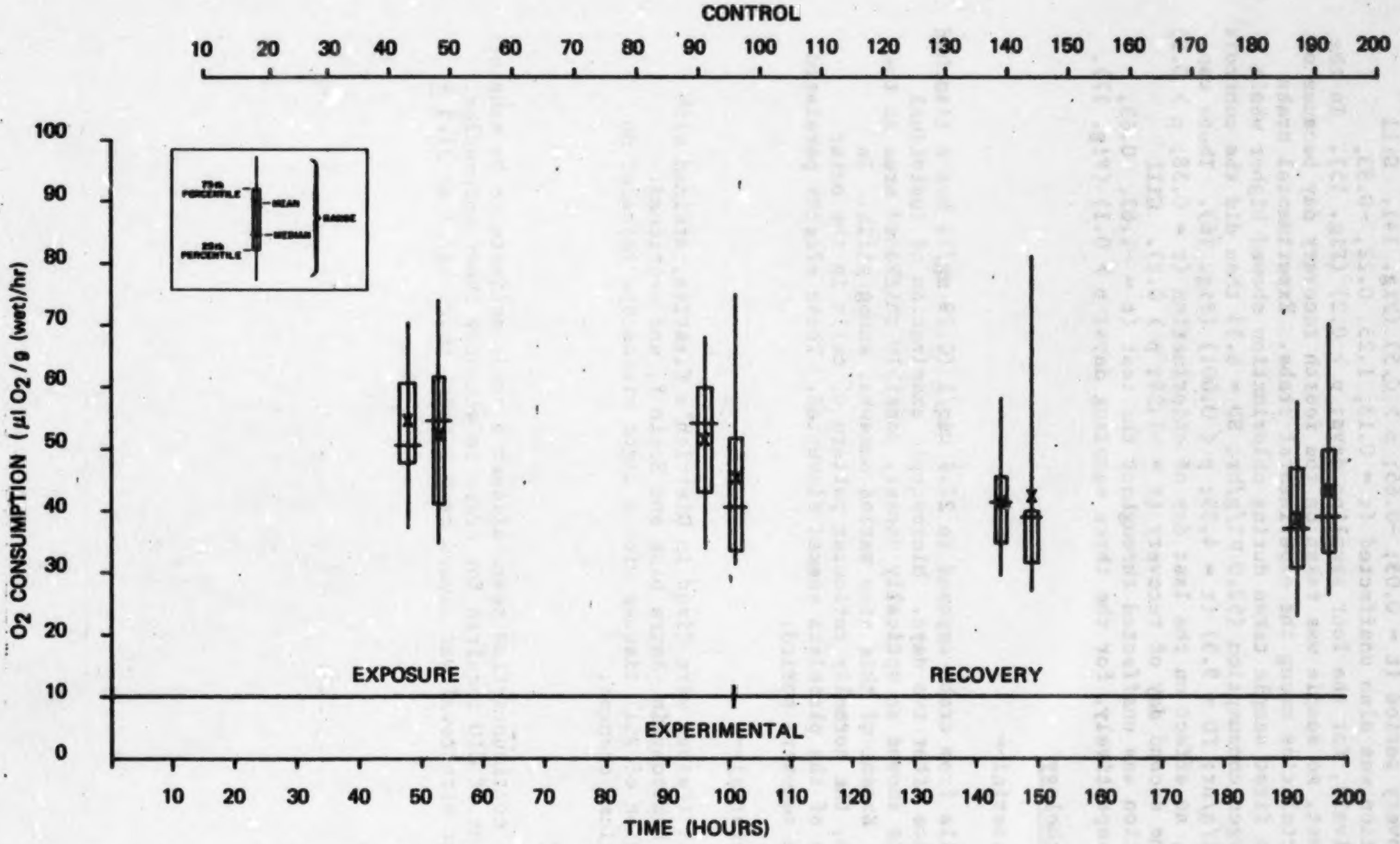

Figure 14. Effects of ClO exposure (0.27 mg $Cl_2/1$) and recovery on whole crab oxygen consumption in *Callinectes sapidus*.

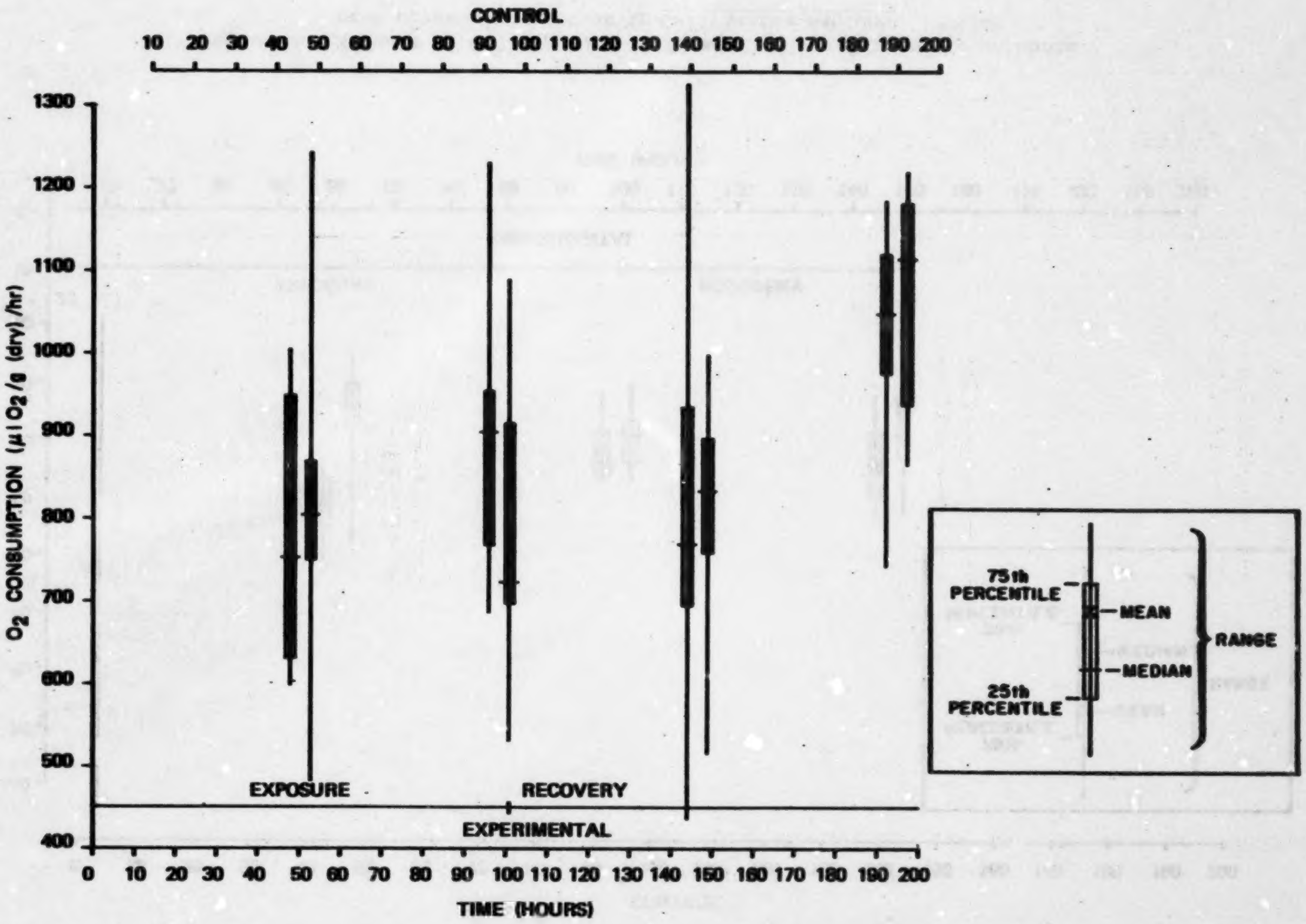

Figure 15. Effects of ClO exposure (0.27 mg Cl_2/l) and recovery on oxygen consumption of excised gills in Callinectes sapidus.

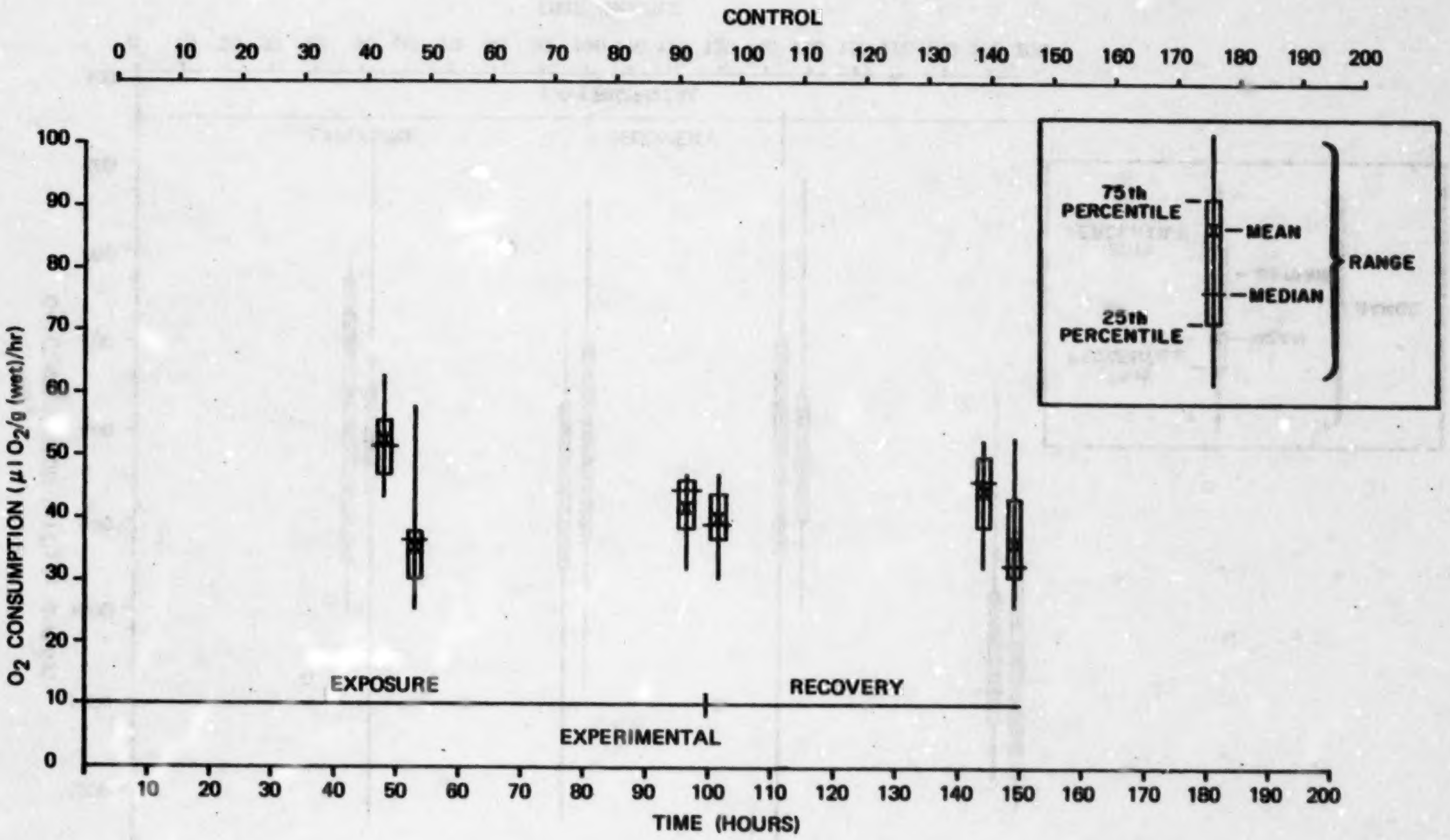

Figure 16. Effects of ClO exposure (0.99 mg Cl_2/l) and recovery on whole crab oxygen consumption in Callinectes sapidus.

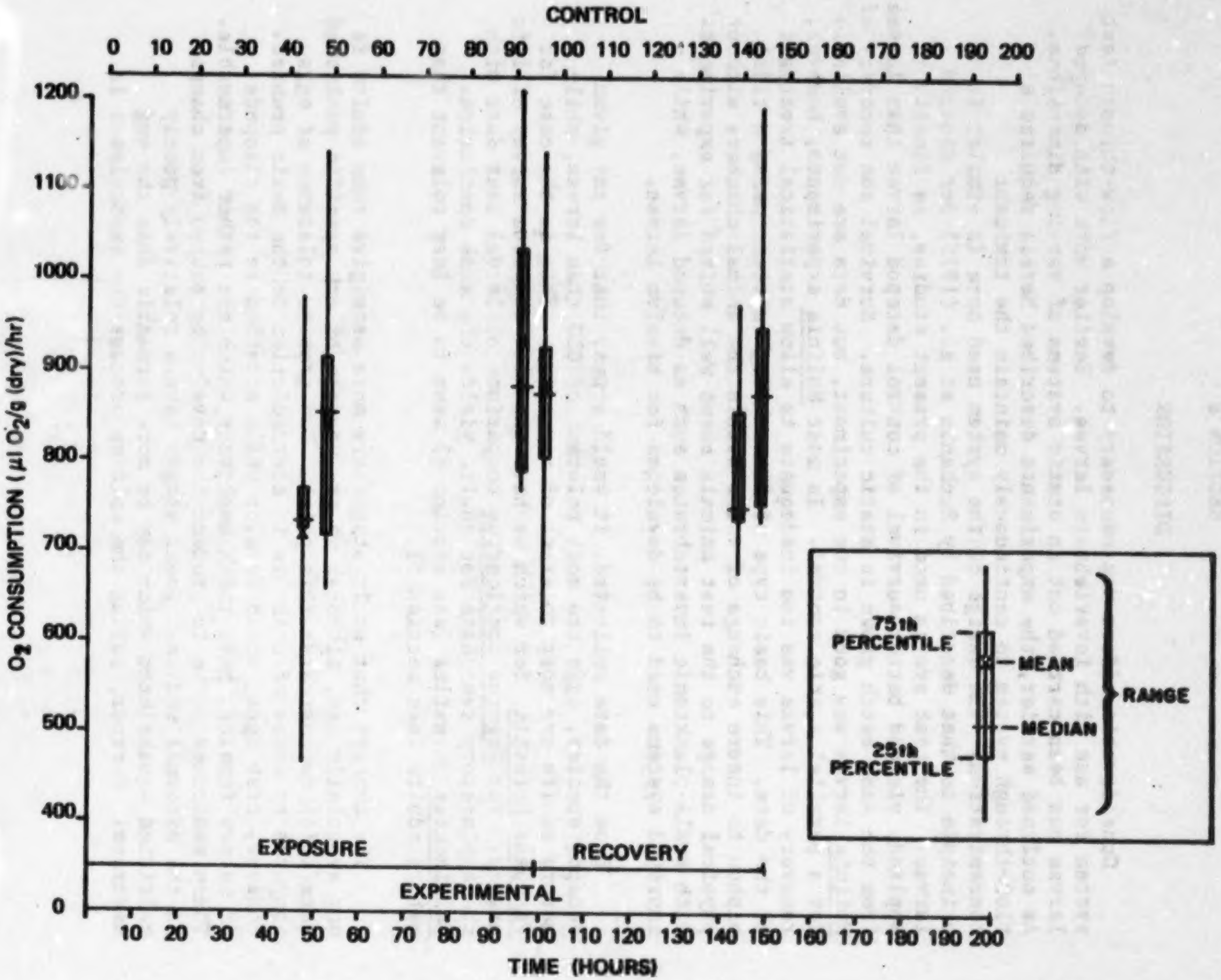

Figure 17. Effects of ClO exposure (0.99 mg Cl_2/1) and recovery on oxygen consumption of excised gills in *Callinectes sapidus*.

SECTION 8

DISCUSSION

Considerable effort was necessary to develop a flow-through test system for use with invertebrate larvae. Earlier work with decapod larvae has been carried out in static systems of varying dimensions. As outlined earlier, the experiments described herein required a flow-through system to continuously maintain the toxicant concentration. The design of the system used here is similar in principle to that described by Buchanan et al. (1975) for decapod larvae. The test system used in the present studies, as finally applied, yielded better survival of control decapod larvae than larvae from the same batch grown in static culture. Survival and recovery of *Mulinia* larvae was good in one experiment, but data are not available for a parallel static control. In most *Mulinia* experiments, however, recovery of larvae was too inadequate to allow statistical treatment of the data. This basic type of flow-through system, using a tidal siphon to insure exchange of water within the animal chambers without physical damage to the test animals seems well suited for experiments with small planktonic invertebrates such as decapod larvae, while improved systems need to be developed for bivalve larvae.

From the data collected, it would appear that for any given decapod species, eggs are more tolerant of CIO than larvae, while mature adults are most tolerant of any stage. This is the case for *Panopeus herbstii*, for which we have the most complete survey of life stages. For *Pagurus longicarpus* comparison of larval test data with the exploratory test data for adults yields the same conclusion. *Callinectes* juveniles (see section 4) seem to be less tolerant than mature adults (see section 6).

The concept that early stages are more sensitive than adults is not especially new, although we are unaware of any specific published data which demonstrates this point. The greater tolerance of eggs compared to zoeas of crabs is in contradiction to the basic premise. However, crab eggs, which develop while attached to the pleopods of mature females, have thick membranes which are rather impermeable. These membranes serve to protect the developing embryo from changes in the external medium. Zoeal stages have a relatively poorly calcified exoskeleton which may be more permeable than the egg membrane. Further, during the molting process the exoskeleton is shed, and water is rapidly taken in before the new exoskeleton hardens. During this period larvae would be especially sensitive to any toxicant. The gut also represents an avenue of entry into larvae for toxicants.

There is an apparent seasonal change in the acutely toxic dose for *Panopeus herbstii* zoeae, with larvae produced early or late in the normal breeding season being more sensitive than those produced during the rest of the breeding season. Tatem et al. (1976) has demonstrated a seasonality in the LC50 of *Palaemonetes* adults exposed to dodecyl sodium sulphate.

Mulinia embryos are less tolerant of CIO than decapod larvae of either species tested. The LC50 for *Mulinia* while ranging from 0.3 to 2.8 µeq/l (0.01 to 0.1 mg/l) was approximately 1.0 µeq/l (0.035 mg/l) in several tests. This value is similar to that derived for oyster larvae (0.73 µeq/l = 0.026 mg/l) using the same test system (Roberts and Gleeson, 1978). For those species tested to date, molluscan larvae appear to be more sensitive to CIO than decapod larvae. Among the crustaceans, only *Acartia tonsa* seems to be as sensitive as molluscan larvae (Roberts and Gleeson, 1978).

Avoidance tests with decapod larvae have not previously been attempted in a flowing-water system, but rather in static systems. The chemical nature of chlorine-induced oxidants necessitated development of a flowing-water test system.

Larvae, when tested under identical conditions of light and temperature in both chambers, respond in the same way, indicating that there is no systematic chamber effect. Salinity does significantly affect the level of response with more positive responses at 18 °/oo than 24 °/oo salinity. This is contrary to previous observations (Roberts, 1971). One would expect the greater response at the higher salinity since 18 °/oo is close to the salinity tolerance limit for larvae. No explanation of the observations can be given at this time. For chlorine avoidance tests, one need not have an explanation. Rather it is only necessary to perform all tests at a controlled salinity(ies).

The effect of light intensity is rather small, with a slightly higher response at 24 fc than at 240 fc. This agrees with the results of Latz and Forward (1977) for *Rhithropanopeus harrissii* larvae. Light should be retained as a variable in chlorine avoidance tests, however, since the measure of response does not measure strength of the response. The slight delay in achieving the response at the lower light intensity suggests that the strength of the response at this intensity is less.

The single test to date yielding information on response to chlorinated seawater, performed with *Palaemonetes pugio*, indicates that larvae can detect CIO at 70.5 μeq/l (2.5 mg/l) as evidenced by telson flips and downward swimming. After a few minutes, however, a few larvae swam into the upper chlorinated layer and stayed there, although they still exhibited signs of distress. This may indicate that the CIO level destroyed or interfered with the chemoreceptors of the larvae. Latz and Forward (1977) reported a similar situation with Rhithropanopeus harrissii larvae challenged by a sudden salinity change. They called this reversal from negative initial responses to positive final response, habituation. However in the study of Latz and Forward (1977) there was no higher salinity layer underlying the salinity layer into which the larvae could retreat. In tests where a refuge layer was provided in previous studies (Lance, 1962; Roberts, 1971), no evidence of habituation in this sense was observed.

The validity of the observation on response to a chlorinated upper layer can only be determined by further study. The minimum CIO concentration which larvae can detect remains to be determined. If habituation is a real phenomenon, it will be necessary to determine whether this occurs at CIO concentrations above the LC50 to assess whether this behavior represents an environmental risk.

Statistically significant as well as apparent trends in serum constituents observed in preliminary tests (experiments 1 through 5) using small sample sizes suggested that exposure to CIO affects the ability of the blue crab to osmoregulate. Effects were measured only at CIO levels at or above the lowest level causing mortality in the test population. Wherever significant effects or apparent trends occurred, chloride and osmotic concentrations decreased and TNPS levels increased in crabs exposed to CIO. Apparent trends for protein were more variable but also tended to decrease overall. All such changes are expected results of osmoregulatory failure in the hyperregulating crabs. Similar effects in the blue crab have been attributed to osmoregulatory failure by Block (1977). Increases in TNPS, in particular, have been related to osmotic stress in Eriocheir sinensis (Vincent-Marique and Gilles, 1970) and Panulirus longipes (Dall, 1975).

Inhibition of osmoregulation was not observed in subsequent tests. Significant effects of CIO on serum constituents occurred sporadically and only in chloride and osmotic concentration. These effects did not occur concommitantly and appeared unrelated to CIO levels. Apparent trends were also highly variable and seemed unrelated to CIO levels. In addition, the majority of trends and statistical effects were in the contrary direction to that which would be expected with failure of osmoregulation in the crabs. This was true even in experiment 12 in which the crabs were originally hyporegulating as a result of the high salinity. Finally, the magnitude of trends and statistical effects was of doubtful physiological significance. Even statistically significant differences in blood chloride and osmoconcentration were rarely over

5% and never more than 10% of control levels.

It is possible that the observed effects were merely secondary manifestations of the impending death of the crabs. Although effects of CIO on serum constituents were of no greater magnitude in experiments with mortalities than in other tests, more pronounced effects were found in moribund crabs.

Since CIO had no clearly apparent direct inhibitory effect on osmoregulation, we felt that the most obvious alternative effect mechanism would be inhibition of oxygen uptake. Initial short-term tests proved promising, showing a general decrease in oxygen consumption of whole crabs with no apparent recovery. However, based on further tests, this effect was probably a short-term reaction to sensing the presence of CIO.

In the remaining tests with whole animals a difference between oxygen consumption of experimental and control animals was observed in only one sample group. In this case exposure to CIO seemed to stimulate oxygen consumption rather than inhibit it after 2 days at 27.9 μeq/l (0.99 mg/l). This effect was ultimately assumed to be spurious, since there were no effects on whole crabs after other exposure intervals or on excised gills from crabs in any tests.

Death of blue crabs exposed to CIO does not seem to be related to asphyxiation. Despite mortalities in the test population exposed to 27.9 μeq.l (0.99 mg/l) CIO, there was no effect on oxygen consumption of surviving whole crabs or excised gills. In addition, the gill damage apparent in fresh material was not confirmed on examination of fixed tissue. Therefore, there is no reason to believe that the ability of blue crab gills to transport oxygen is permanently inhibited by exposure to CIO.

There appeared to be no relationship between the occurrence of mortalities among test populations and effects of CIO on serum constituents and respiration of crabs from these populations. Mortalities in many tests occurred in the absence of effects on serum constituents and respiration. This fact coupled with the magnitude and the sporadic nature of effects observed in some tests leads to the conclusion that there are no physiologically significant sublethal effects of CIO on the selected serum constituents or oxygen consumption of whole blue crabs or excised gills.

We observed an obvious sensitivity of blue crab antennules to CIO and inhibition of spawning and feeding in dosed crabs. The effect on spawning occurred at CIO levels as low as 12.4 μeq/l (0.44 mg Cl_2/l), and the effect on feeding was at CIO levels as low as 10.2 μeq/l (0.36 mg Cl_2/l). Although the effects seem to be transient (as observed in antennule activity and feeding response), disappearing when CIO is no longer present, they have long term implications and should be considered in determining safe levels of CIO for blue crabs. It would be useful to test whether these effects ever become permanent and to quantify more precisely their relationships to dose and time.

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